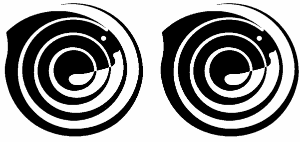
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Port Curtis Mud Crab Shell Disease - nature, distribution and management

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FRDC Project No. 98/210

Leonie Andersen and John Norton

Central Queensland University ? Queensland ? Gladstone ? 2001

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PROJECT #98/210
Port Curtis mud crab (*Scylla serrata*) shell disease; nature, distribution and management.

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Objectives:

1. Define the histologic stages of the lesion by developing a sequence of pathological events (includes pathology tests).
2. Define the epidemiology
a) Define the prevalence and distribution of the disease in each age group in Port Curtis. Determine if the same disease occurs in other areas.
b) Determine if affected crabs are able to moult and therefore mate successfully by experimentally observing different combinations of diseased male/female crabs. Determine if the crab can shed the ulcerations during a moult. Is there a healing stage?
3. Cross infection: if an infectious agent is isolated/identified, determine its ability to cross infect other species of crustaceans.
4. Depending on the cause/s isolated/identified, work out a management strategy to lessen the effects of rust spot shell disease.
5. Investigate metal burdens of mud crabs from Gladstone compared to other areas.
6. Through the use of copper exposure trials, investigate exposure to metals (in particular copper) as a possible cause of rust spot lesions.

Non Technical Summary:

Outcomes Achieved:
The project outcome that rust spot shell disease in Port Curtis is not infectious has allayed the concerns of commercial and recreational fishing sectors as well as the aquaculture industry, as to the potential impact a contagious disease could have had on these industries. As the project determined that marketability was not affected by gross shell ulcerations, community confidence in the local mud crab fishery has been restored. Although elevated metal concentrations in mud crab tissues are a concern, consumers have been assured that consumption does not pose a potential health risk.

The elevated metal concentrations have, however, raised community awareness of ecosystem health issues, which can occur where there is an interface of urbanisation and fishing habitats. The Queensland Seafood Industry Association has welcomed the release of the results as they highlight the need to maintain a healthy marine environment. The project finding that mud crabs could be "stressed" has alerted managers and relevant agencies to examine current environmental performance indicators. The project has also created extensive knowledge of the epidemiology of shell disease and mud crab biology as a whole.

Commercial fisherman first noticed rust spot shell lesions in the Portunid mud crab (*Scylla serrata* - Forskal) in Gladstone Harbour, Port Curtis Queensland in 1994. The irregular shaped orange coloured lesions commonly called "rust spots" were located on the dorsal shell of the mud crab and had the appearance of cooked crab shell. In advanced cases lesions perforated to form an ulcer, often exposing internal organs. Concerns were raised not only for the potential impact on mud crab marketability, but the possibility that the shell lesions could spread to other crustaceans, in particular the lucrative prawn aquaculture industry. In order to define the disease syndrome, a number of possible causative or contributing factors were examined in the course of the disease investigation. These included infectious/non-infectious causes, environmental factors and contaminant loads.

Over 3000 mud crabs from a number of locations in Queensland (Port Curtis, Ayr, Jacobs Well and Fitzroy River) have been examined for the presence of rust spot shell lesions between 1998 and 2001. The total prevalence of shell lesions in Port Curtis was lower in the 1998/99 sampling (18.3%) compared to that in 2001 (10.2%). Indications from archived crab shells suggest that the prevalence of lesions was much higher when the syndrome was first recorded around 1995. Future samplings should be continued, however, to establish if the decreasing trend continues. There was no significant difference in the prevalence in Port Curtis compared to Fitzroy River, which is possibly due to intermixing of crabs from these two locations. Although lesions occur in mud crabs from Jacobs Well (Moreton Bay) and Ayr, the prevalence is low (from 0-5.6%) compared to Port Curtis. None of the juvenile mud crabs examined from Port Curtis had shell lesions.

A lesion grading system was designed to assist in accurately documenting the area of the shell affected and the severity of lesions. Although females had a higher prevalence of shell lesions than males in Port Curtis in the 1998/99 sampling, lesions occurred in equal frequencies in both sexes in the 1999/00 sampling. Females did, however, have a higher frequency of larger non-perforated lesions than males. There was also a gender difference in the area of the carapace to which lesions were distributed, which may be related to reproduction and spawning in adult females. A majority of affected crabs had two lesions, which were bilaterally symmetrically located on the carapace in over 50% of cases. Less than 10 % of all lesions examined were perforated or ulcerated. As less than 1% of the crabs marketed or consumed from Port Curtis have perforated lesions, it is unlikely that marketability has been affected by the presence of the rust spot syndrome.

Over 60 mud crabs with rust spot lesions were chosen for histopathological examination and the results compared to a reference group of 30 non-diseased mud crabs. There was no evidence of an infectious or parasitic agent being associated with any internal organ or with the carapace lesions. The pathology of the rust spot lesions is restricted to the endocuticle layer (internal shell layer) and adjacent muscle attachment. As this layer is formed after the crab has moulted, it appears that the lesions are caused by a defect in the manufacturing of this layer, while the crab is in the process of calcifying its shell. This pathology contrasts with previously reported pathology of shell diseases in other crustaceans. Here the pathology is an external erosion of the shell, which may be caused by pathogenic organisms, with unsuitable environmental conditions being a contributing factor.

Cross infection trials were conducted in an attempt to transmit rust spot shell disease to other mud crabs, sand crabs and prawns. In water transmission trials, adult and juvenile mud crabs were exposed to Gladstone Harbour water. In inoculation trials, processed tissue from diseased mud crabs was injected into juvenile mud crabs, sand crabs and prawns. None of the treatment groups developed rust spot lesions and after subsequent pathology, no significant lesions were seen in any of the tissues examined. It appears that rust spot lesions do not contain a virus or virus-like organism capable of transmitting the disease to other mud crabs or crustaceans. It is probable that the cause of rust spot shell lesions is non-infectious and possibly environmental.

A number of diseased mud crabs both adult and juvenile was observed through a moulting period in order to determine if lesions could be shed with the old shell when the crab moulted. Old lesions were shed with the old exuviae (shell) and a healed form of the lesion remained in some cases. New lesions were also seen to form in the post moult phase (during production of the new endocuticle) and would most likely remain until the next moult. Although diseased mud crabs even with severe lesions are able to moult successfully and repair shell lesions, in some cases where lesions are extensive, however, they may contribute to the cause of moult death syndrome.

Metal analyses of 220 mud crab tissues were undertaken in 1999 and 2000. Metal concentrations, in particular copper and zinc, were elevated in Gladstone (and Fitzroy River) mud crab hepatopancreas (liver), compared to a reference site (Ayr). There was no significant difference in metal concentrations between the diseased and non-diseased group of mud crabs from Gladstone. The inability to establish differences, however, could be confounded due to the difficulty in assigning a crab to either of these groups being qualitative (the presence of a lesion) rather than quantitative. Concentrations of copper in Gladstone mud crab hepatopancreas were also up to three times higher than concentrations in the hepatopancreas of mud crabs from other locations sampled in Queensland. Metal burdens in Gladstone mud crab hepatopancreas were also elevated compared to Ayr. A high variation in metal concentrations in the Gladstone diseased group of crabs compared to all other groups suggests that these crabs are unable to regulate their metal concentrations and this could indicate some level of stress in this group. Concentrations of all metals in muscle tissue were below those metal concentrations recommended by the Australia New Zealand Food Standards Code (2000) for the consumption of crustacea. This code is continually under review and therefore metal concentrations in mud crab tissues should continue to be monitored. However, in terms of metal concentrations mud crab meat from Gladstone crabs can be considered suitable for consumption.

Through the use of copper exposure trials in which juvenile mud crabs were exposed to sublethal levels of copper, we explored the hypothesis that copper exposure inhibits calcium uptake into the post moult crab shell and could therefore be implicated in the development of rust spot shell lesions. The trial confirmed that calcium uptake into the carapace (shell) of soft-shelled crabs (72 hours post moult) was inhibited by sublethal copper exposure. There was a significant negative relationship between increasing copper concentrations in the hepatopancreas and declining calcium concentrations in the carapace. Several metals including copper have been shown to cause interference with calcium uptake in crustaceans. It is therefore conceivable that exposure to copper, perhaps in combination with other metals/contaminants could be implicated in the cause of rust spot lesions.

Results of crab blood tests in which two immune parameters and one cellular enzyme were measured, suggest that Gladstone crabs have been stimulated to produce higher levels of these immune/cellular factors compared to the Ayr crabs. This could further indicate a higher level of stress in Gladstone crabs compared to those from Ayr. Exposure to pathogens, contaminants and stress are known to effect production of immune factors in aquatic organisms. Although immune factors/cellular enzymes in the female diseased group of crabs from Gladstone were elevated compared to the female crabs from Ayr, they were significantly lower than levels in the non-diseased female crabs from Gladstone. This suggests that production of immune factors/cellular enzymes in this diseased group may have been suppressed or the factors have been destroyed at a faster rate than normal.

Stepwise multiple regressions were used to investigate whether any relationships existed between measures of blood immune/cellular responses and hepatopancreas metal concentrations in Ayr and Gladstone crabs. Results suggest that there is a statistical relationship between the metal concentrations and immune/cellular responses. There were fewer, weaker relationships identified in the Ayr crabs in comparison to the Gladstone groups, although at times relationships in the Gladstone crabs were inhibitory rather than stimulatory (i.e. indicating exposure to a metal causing inhibition rather than stimulation of a blood parameter). Although the regressions do not prove cause and effect they do, however, provide proof of a relationship, whereby blood parameters can change as a function of a change in metal accumulations in Gladstone mud crab tissues.

The results of metal analyses of water and sediments from the permanent burrows of Gladstone mud crabs, indicate that fairly low concentrations of metals exist in the burrows and therefore these sites are unlikely to be a source of elevated metals in Gladstone mud crabs. A pilot study using stable isotopes of carbon and nitrogen as an alternative to gut content analyses was used to determine if differences existed in the diets of Gladstone crabs compared to Ayr crabs which, might explain the contrasting tissue metal results between the two sites. The results although preliminary, suggest that the Gladstone crabs may be consuming something in their diet, which is enriched in copper but is not available in the Ayr mud crab diet.

Our findings have raised questions as to the comparative “health” of Gladstone crabs compared to Ayr and hence the quality of the ecological environment of Port Curtis. As the cause of mud crab rust spot shell lesions appears to be a local environmental one, the source of elevated metal concentrations especially copper and zinc, needs to be investigated further. The impact of elevated metal burdens and stimulated immune responses and cellular enzymes on individual mud crabs is not known. The Centre for Environmental Management (CQU) is conducting two small research projects to determine metal concentrations in other biota in Port Curtis which may be part of the mud crab diet. There is, however, an urgent need for research to go beyond the food sources of the mud crab, to the origin of the contamination of those organisms eg. urban, industrial or agricultural.

Our recommendations are:

1. To establish if elevated metal concentrations exist in other biota in Port Curtis.
2. Determine the source of elevated metal concentrations.
3. Reduce the flow of metals into Port Curtis from all sources by reviewing the current point and area sources of discharge.

4. Continue to monitor crab health in terms of the prevalence of rust spot shell lesions and metal burdens, ensuring metal levels remain within recommended levels.

Further research into the ecosystem health of Port Curtis will determine what management strategies are required to address this issue.

CHAPTER 1. General Introduction

1.1 BACKGROUND

Commercial fishermen first noticed rust spot shell lesions in the Portunid mud crab (*Scylla serrata* – Forskal) in Gladstone Harbour, Port Curtis Queensland in 1994 (Figure 3.4). Gladstone is an industrial city adjacent to a deep harbour (Port Curtis), an international trades port, which also supports a large commercial and recreational fishing industry (QDEH 1994). This previously unreported shell disease had the potential therefore to damage the lucrative Queensland mud crab market worth approximately AUS $7 million per year (Williams 2000). The irregular shaped cuticular lesions commonly called "rust spots" are confined mainly to the dorsal carapace and appeared initially as well circumscribed orange-coloured areas, similar to that of cooked crab shell (figure 1.1). In advanced cases, affected areas of the cuticle would deteriorate to form an ulceration often exposing internal organs (figure 6.7). Without an intact carapace the mud crab is exposed to invasion by pathogenic microorganisms, which could cause bacterial septicaemia and eventual death.

Figure 1.1. Female adult mud crab with a number of non-perforated carapace rust spots, one of which is circled.

Shell disease has been reported in many crustaceans of economic importance (Sindermann 1989a), in association with a variety of environmental conditions (Noga 1991). The pathogenesis of shell disease is thought to be multifactorial and strongly influenced by mechanical damage to the cuticle, degradative activities of invading bacteria (Cook and Lofton 1973, Baross et al. 1978, Malloy 1978) and fungi (Alderman 1981) and external factors including water and soil contaminants, low dissolved oxygen and high nutrient loads (Young and Pearce 1975, Engel and Noga 1989, Sindermann 1989b). Bacterial shell disease is common in impounded crustaceans and is thought to be facilitated by environmental stressors, such as overcrowding and poor water quality (Prince et al 1993).

The initial prevalence of rust spot shell disease as reported by commercial fisherman at that time (1994/1995), was estimated to be around 10% although the exact extent of the disease was not known. Concerns were raised not only for the health of the crab in terms of morbidity and mortality, but also because of the effect of unsightly, gross ulcerations on marketability. Adhesions formed under ulcerations could also inhibit moulting of the old shell and therefore reproduction and growth. These latter complications would have detrimental impacts on mud crab populations as a whole. Deleterious effects could also extend to the recreational sector due to the public's perception that the mud crabs were not "safe" to eat.

A pilot investigation into the disease was instigated in 1996 by the Department of Environment through their scientific assessment branch in Brisbane. This investigation was aided by limited funding but generous support from the local community and industry. Although a range of toxicological tests were undertaken on both mud crabs and sediments, the results were inconclusive. One of the main concerns was that the cause of the disease was infectious and its introduction to the area may have been via ballast water. If the pathogen was an infectious agent, there was the possibility of cross infection to other crustacean species, in particular the important prawn aquaculture industry.

1.2 NEED

An outbreak of a previously unrecorded shell disease in this important Queensland icon was of concern not only to the Gladstone community but also, to the fishing industry as a whole. Questions were raised about the possible introduction of a new disease and also the eco-health status of the harbour in this rapidly growing industrial city. To determine the magnitude of the outbreak, the prevalence and geographical extent of the disease needed to be established and documented. Therefore by monitoring the prevalence of the disease in Gladstone and other areas, predictions could be made about its likely spread. The stages of the life cycle (juveniles or adults) that were susceptible to contracting lesions would also have an impact on future management strategies.

A grading system was required in order to systematically and consistently document lesions. The extent and severity of lesions would evaluate the exact effect lesions would have on marketability. By defining the histology of progressive stages of lesions, a sequence of pathological events could be established and the cause/s if pathogenic, identified. Necropsy and pathology of both diseased and non-diseased crabs would help determine if the syndrome was infectious or non-infectious and if the disease had effects on other organs or systems other than the shell.

Through transmission trials the infectivity and virulence of a potential pathogen could be established, thereby determining the morbidity and mortality of the disease under controlled conditions. By involving other species in transmission trials the potential of an infectious agent to cross infect other crustaceans could be established. This aspect was important not only for commercially significant species but also for other crustaceans, which are keystone species in mangrove communities.

Trials involving diseased moulting crabs would determine if adhesions formed by lesions could prevent the crab from shedding its shell at successive moults. As crustaceans must shed their shell in order to grow, the inhibition of the moulting

process could have a large impact on stocks. Mating also only occurs when the female mud crab is in the post moult, soft shell form, immediately after shedding her shell. Therefore interference with mating could lead to reduced spawning.

As the trophic position of mud crabs is relatively high in the food chain, the bioaccumulation of contaminants required consideration. As shell disease in other crustaceans had been previously linked to polluted environments, the contaminant loads of Gladstone mud crabs required taking into account.

The introduction of management strategies to combat rust spot syndrome can only be made once the aetiology and epidemiology of the disease have been established. The part that secondary factors, particularly environmental, play in the progression and spread of the syndrome needed to be determined. Once the extent of rust spots shell lesions had been developed a management plan to reduce the negative impact on both wild and cultured crustaceans, could be implemented.

1.3 OBJECTIVES

The original objectives of the project were to:

1. Define the histological stages of the lesion by developing a sequence of pathological events and isolate the cause/s.
2. Define the epidemiology
   a) Define the prevalence and distribution of the disease in each age group in Port Curtis. Determine if the same disease occurs in other areas.
   b) Determine morbidity/mortality rates in selected age groups by controlled experiments. Determine if there is any affect on growth.
   c) Determine if affected crabs are able to moult and therefore mate successfully by experimentally observing different combinations of diseased male/female crabs. Determine if the crab can shed the ulcerations during a moult. Is there a healing stage?
3. Cross infection:
   If an infectious agent is isolated/identified, determine its ability to cross infect other species of crustaceans
4. Depending on the cause/s isolated/identified, work out a management strategy to lessen the effects of rust spot shell disease.

1.4 REVISED OBJECTIVES

During the course of the research and from the results that were obtained from the transmission trials, it became apparent that the cause of the disease appeared to be non-infectious and probably environmental. Therefore some objectives were altered and more objectives added to accommodate the changes in the direction of the research.

1. Define the histologic stages of the lesion by developing a sequence of pathological events (includes pathology tests).
2. Define the epidemiology
   a) Define the prevalence and distribution of the disease in each age group in Port Curtis. Determine if the same disease occurs in other areas.

   b) Determine if affected crabs are able to moult and therefore mate successfully by experimentally observing different combinations of diseased male/female crabs. Determine if the crab can shed the ulcerations, during a moult. Is there a healing stage?
3. Cross infection
   If an infectious agent is isolated/identified, determine its ability to cross infect other species of crustaceans.
4. Depending on the cause/s isolated/identified, work out a management strategy to lessen the effects of rust spot shell disease.
5. Investigate metal burdens of mud crabs from Gladstone compared to other areas.
6. Through the use of copper exposure trials investigate exposure to metals (in particular copper) as a possible cause of rust spot lesions.

CHAPTER 2. A Disease Investigation

2.1 INTRODUCTION

When presented with an investigation of an animal disease, the focal point of that investigation is to make a diagnosis. 'Disease' can be defined as 'inability to perform physiological functions at normal levels provided nutrition and other environmental requirements are provided at adequate levels' (Blood et al. 1983). The clinical examination not only involves the affected animal/herd, but also includes a history and the environment. Therefore the following elements were taken into account in the differential diagnosis of rust spot shell disease and their potential association with the syndrome, considered.

2.1.1 Case History

As shell disease was first noticed in Gladstone mud crabs in 1994, a search was made into the history of Gladstone over the last 10 years as to whether any significant changes had occurred in the area, which could be related to the outbreak of rust spot shell disease.

Meteorology – Rainfall data received from the Bureau of Meteorology for the Gladstone area show that there was a much lower rainfall in 1993-1995 (mean approximately 596mm) compared to the preceding three years 1990-1992 (mean approximately 987mm). If the onset of shell lesions was related to contaminants it is possible that the lack of flushing of the environment due to significant rainfall events, may have been a contributing factor. There have been no significant effects from tropical cyclones in the area in the last 10 years. Fluctuations in air temperatures have also remained consistent over this time.

Population – The population of Gladstone in 1994 was 25 781 and has increased slowly at an average annual growth rate of 1.3% (ABS 2001). Therefore no major growth explosions have been recorded in this time.

Industry – There are seven major industries in the Gladstone area and a number of smaller industries, all of which have licences to discharge under the EPA. No new major industries, however, have began production between 1990 and 2000.

2.1.2 Infectious/non-infectious

The possibility that the disease syndrome was caused by an infectious agent was foremost in our list of differential diagnosis. The results of the pathology of internal organs and of transmission and inoculation trials are dealt with in Chapter 4 & 5 respectively.

2.1.3 Contaminants

A number of contaminants were considered as being potentially implicated in the disease syndrome. Metal levels are discussed in Chapter 7.

Pesticides – Samples of Gladstone mud crab tissue analysed in 1996 for DDT, Dieldrin and Heptachlor epoxide recorded fairly low levels compared to crabs from other sites (Brisbane area and Maroochy River). Levels were also below ANZFA (1999a) food code limits Mortimer (2000). As industry rather than agriculture forms the key economic resource for the area, pesticides were not considered to be a major problem.

Dredging – The harbour is dredged by the Gladstone Port Authority and the dredge spoils dumped at sea. Routine analyses of dredged sediments for trace elements, organochlorine pesticides, polynuclear aromatic compounds, polychlorinated biphenyls and organotin compounds have found that sediments from arbitrary dredging blocks were uncontaminated (WBM 2000).

Ballast water – Gladstone is a large industrial port, which had an annual throughput of approximately 23.9 million tonnes in 1992/1993 and has steadily increased to 42.8 million tonnes in 1998/99 (GPA 2000). Although ballast water is a possible source for the introduction of marine pests, the Australian Quarantine and Inspection Service introduced guidelines in 1990 aimed at minimising this risk. It is thought that about 80% of ships entering Australian water comply with these guidelines (QDEH 1994). A recent survey of Port Curtis for introduced marine species detected the following introduced species during the survey or collected in survey samples: the ascidians *Styela plicata* and *Botrylloides leachi*; the bryozoans, *Amathia distans*, *Bugula neritina*, *Cryptosula pallasiana*, *Watersipora subtorquata*, and *Zoobotryon verticillatum*; the hydrozoan *Obelia dichotoma* and the isopod, *Paracerceis sculpta.* A dinoflagellate *Alexandrium sp.* was also detected in samples. This was definitely not the target species *A. tamarense*, *A. minutum* or *A. catenella*, but is likely to be a non toxic *A. affine*, which has been located elsewhere in Australia (Lewis et al 2001), none of which have been associated with shell disease. The toxic dinoflagellate, which is known to cause skin ulcers in fish (Noga et al 1996a) was not identified.

Tributyltin (TBT) – TBT has been known to cause anomalies in shell calcification in adult oysters (Alzieu 1998) and to also enhance the uptake of copper by a synergistic relationship (Batley et al.1992). Sediments analysed for TBT in 1996 and 2000 by the Gladstone Port Authority, however, were considered to be within the ANZECC 1998 Interim Ocean Disposal Guidelines screening levels (WBM 1996, 2000). Hepatopancreas tissue samples from five Gladstone diseased, five non-diseased and five Ayr female mud crabs collected in 2000 were analysed for butylin levels. Although sample numbers were small and variation within groups of levels of tributylin was high, there was no significant difference among the groups (df = 2,12 p = 0.349). Levels of TBT in Gladstone ranged from <1 –238 ng Sn/g and in Ayr from <1 – 58 ng Sn/g wet wgt[?]. As oysters are the preferred species for monitoring TBT levels, little data exists on TBT levels in crustaceans. Kannan et al. (1995), however, recorded 2200 ng Sn/g of TBT in Horseshoe crab (*Tachyplues tridentatus)* hepatopancreas from Habu Bay, an industrial harbour in Japan. Levels of 115 ng Sn/g were also recorded from a crab from a more oceanic site (Hakata Bay). Total hepatopancreas butylin concentrations from the 6 crabs sampled at each site ranged from 570–5000 ng/g in Habu bay and 350–2270 ng/g in Hakata Bay. Therefore levels of tributylin in mud crab hepatopancreas from Gladstone or Ayr do not appear to be elevated in comparison to this study. Kannan et al. (1995) also did not record any instances of shell disease in the crabs they studied.

The use of TBT-based antifouling paints on vessels under 25m has been banned in Australia since 1989 and in some other countries prior to this date. As the half life of TBT in seawater is relatively short (around 6hrs) (Batley 2000), it is possible that levels of tributylin in mud crab hepatopancreas in 1994 at the time of the recorded

[?] Data obtained in collaboration with Mary-Anne Jones, CRC Masters student, CQU Rockhampton.

appearance of rust spot shell disease, was much higher than levels recorded in 2000. Port Curtis is a relatively new shipping harbour with a small area of shipping movement and possible contaminant area, relative to the total size of Port Curtis mud crab habitat. Although TBT could be a contributing factor along with a range of contaminants, which may be impacting on Gladstone mud crabs, it is not considered to be a direct cause of rust spot shell disease.

Antimosquito agents – Two antimosquito agents have been recorded as causing cuticular defects in crustaceans. Diflubenzuron caused shell lesions on regenerating limb buds of the fiddler crab (*Uca pugilator*) (Weis et al 1987) and Methoprene at a concentration of 10µM caused disruption in shell formation in the blue crab (*Callinectes sapidus*) (Horst and Walker, 1999) in laboratory trials. Difluenzuron, however, is not used in Australia. Although Methoprene was first used by the Gladstone City Council in 1995 it is used infrequently and the low environmental concentrations are unlikely to cause the same effects as those seen in laboratory trials.

Conclusion

In order to define the epidemiology of the shell disease syndrome in Gladstone, a case history highlighting factors included in the differential diagnosis has been developed. Factors included infectious/non-infectious, environmental, meteorological and contaminants. A number of these factors are discussed in further Chapters.

CHAPTER 3. The Prevalence of Shell Disease

Objectives: *To define the prevalence and distribution of the disease in each age group in Port Curtis and to determine if the same disease occurs in other areas.*

3.1 METHODS

3.1.1 Sampling- Adults

From October 1998 to April 1999 and December 1999 to April 2001 inclusive, 3008 adult (for the purpose of this study, crabs greater than 120mm carapace width) mud crabs (*Scylla serrata*) from a number of locations in Queensland were examined for the presence of cuticular lesions. Crabs were either caught by our research team using baited standard mesh crab pots (Figure 3.1), or supplied by commercial fisherman. Crabs were examined within 24 hours of capture and evaluated for the presence of rust spot lesions. Examination included sexing and measuring the carapace width of a sample of crabs from each site and recording details of any carapace lesions. Only dorsal carapace lesions were recorded, as rust spots rarely involved the appendages, abdomen or ventral thorax. Lesions were then graded according to size and colour and whether they had perforated the surface of the cuticle. The distribution of lesions was allocated to specific regions on the carapace in order to determine whether there was a link between the areas in which lesions occurred and the underlying soft tissues.

Figure 3.1 Deployment of crab pots in Port Curtis

Seven sampling sites were established in Port Curtis for the 99/00 crab season (Figure 3.2). However, as no male crabs were caught at Site 6, this site was later disregarded. Sites were chosen in order to establish whether the occurrence of lesions in different areas of the harbour suggested anthropogenic effects on the prevalence of disease. The sites were sampled in 2000 on at least two occasions in either December, March and

June, to determine if there was a seasonal influence on the occurrence of crabs with lesions. Results of the 1375 adult crabs caught in from Port Curtis 99/00 were then compared statistically with the results from the 645 crabs examined in 98/99 and the 309 crabs examined in 2001. Mud crabs were also examined from Jacobs Well (Moreton Bay) (n=249), Ayr (North Queensland) (n=101) (Figure 3.3) and Fitzroy R. (Rockhampton)(n=329)(Figure 3.2).

3.1.2 Sampling- juveniles

In January and February 2000, 63 male and female juvenile and adolescent mud crabs ranging from 12mm to 120mm carapace width were caught in upper estuarine mangrove areas of Port Curtis, using baited mesh bait pots (Figure 3.4). Crabs were examined at the site or within 24 hours of capture and processed as for the adult crabs above.

Figure 3.4 Setting bait pots for juvenile mud crabs

Figure 3.3 East Queensland coast showing sampling locations for mud crabs. (Adapted from Mortimer 2000)

3.1.3 Lesion grading system

The grading system developed to describe the gross severity of rust spot lesions is outlined in Table 3.1. The system is based on the size of the lesion in terms of area and whether the epicuticle had been perforated. The numbered areas of the carapace to which lesions are allocated are depicted in Figure 3.5.

Table 3.1 Gross grading system for rust spot lesions in mud crabs.

Grade 1:	Non Perforated $< 5mm^2$
Grade 2:	Non Perforated > or = $5mm^2$
Grade 3:	Perforated cuticle (either partially or fully) $< 5mm^2$
Grade 4:	Perforated cuticle (either partially or fully) > or = $5mm^2$ & $< 20mm^2$
Grade 5:	Perforated cuticle (either partially or fully) > or = $20mm^2$

Figure 3.5 Numbered areas of the mud crab carapace to which lesions are allocated.

1.1.1.1.1

3.2 RESULTS

3.2.1 Lesion prevalence in Port Curtis

The gender difference in prevalence of male and female mud crabs with rust spot lesions in Port Curtis 98/99 and 99/00 and 2001 crab collections are presented in Figure 3.6. There was a gender difference in the proportion of females with lesions (23.7%) compared to males (9.9%) in 98/99 (Chi-squared (?^{2}) = 18.4938, df = 1, $p < 0.0001$). However, lesions occurred in equal frequencies in females (13.1%) compared to males (15.9%) in 99/00 (?^{2} = 1.4245, df = 1, p = 0.2327). Although a significantly lower proportion of total crabs had lesions in 99/00 (14.5%) compared with 98/99 (18.3%) (?^{2} = 4.0663, df = 1, p = 0.0437), the gender difference had changed in 99/00 with a higher proportion of males with lesions in 99/00 (15.9%) than in 98/99 (9.9%)(?^{2} = 4.5492, df = 1, p = 0.0329). In contrast the proportion of females with lesions in 99/00 (13.1%) decreased compared to 98/99 (23.7%) (?^{2} = 16.6191, df = 1, $p < 0.0001$). Although there was a decrease in the proportion of diseased crabs compared to non diseased in 2001 (10.2%) compared to 99/00 (14.5%), the decrease was not significantly different (?^{2} = 1.756, df = 1, p = 0.185). There was, however, a significant decrease in disease prevalence in 2001 compared to the 98/99 sampling (?^{2} = 6.974, df = 1, p = 0.008). The total prevalence of mud crabs with rust spot shell lesions from Port Curtis is presented in Figure 3.7.

Figure 3.6 The Proportion of female diseased crabs compared to female non-diseased crabs and the proportion of male diseased crabs compared to male non-diseased crabs in Port Curtis in 98/99, 99/00 and 2001 sampling occasions.

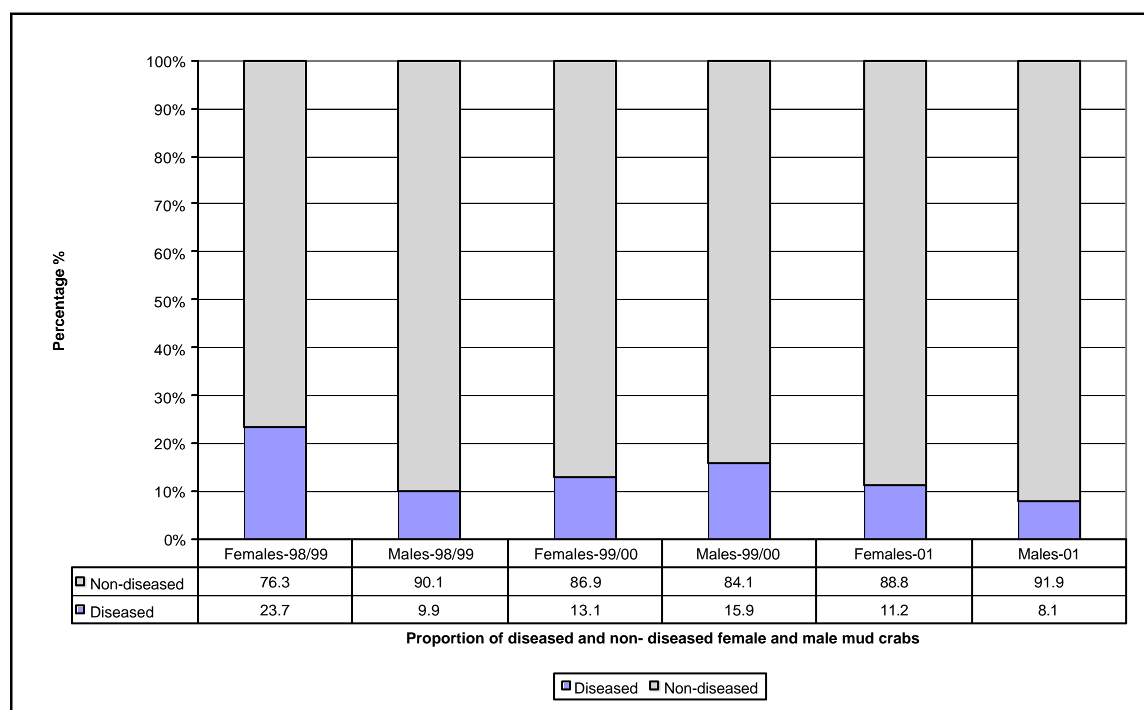
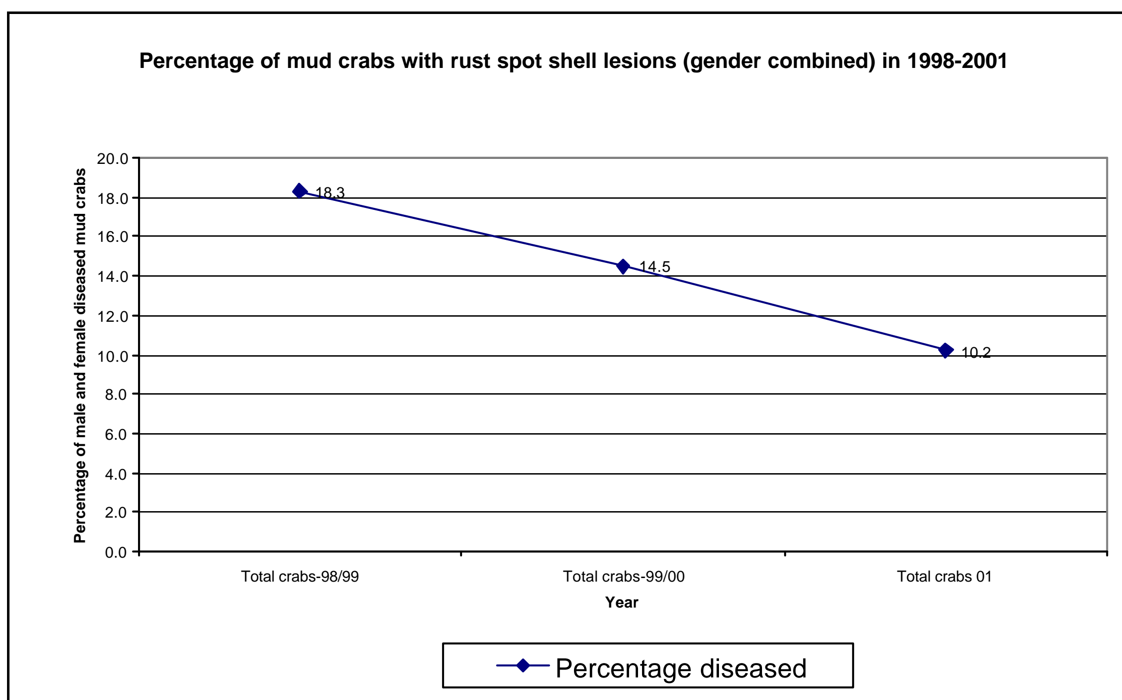


Figure 3.7 The total prevalence of rust spot shell disease in Port Curtis over the three sampling occasions as a percentage of the total number of crabs examined.

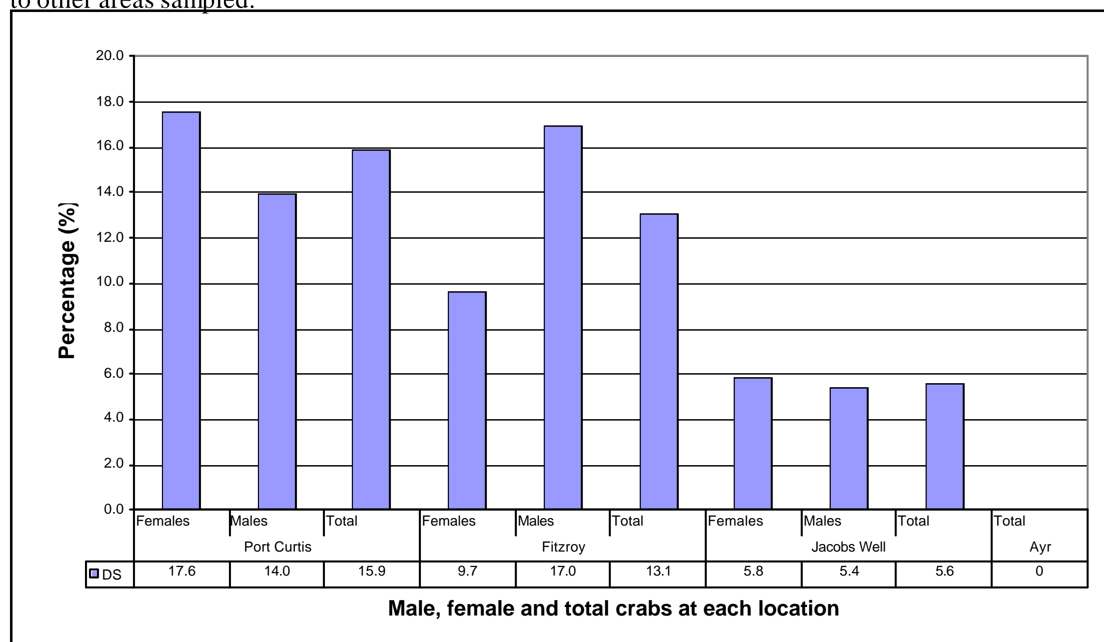


The March 2000 data for lesion prevalence from the six different sites within Port Curtis were compared to determine if the results suggested possible anthropogenic influences on prevalence of lesions in the harbour. There was no difference in the frequency of lesions at the different sites ($?^2 = 8.7658$, df = 5, $p > 0.05$). As there was no site difference in the prevalence of lesions, the data were pooled for each time period to determine if there was a seasonal influence on lesion prevalence. There was a significant difference between time periods with a relatively higher prevalence of disease in December and a relatively lower prevalence in June ($?^2 = 13.2868$, df = 2, $0.01 > p > 0.001$).

3.2.2 Lesion prevalence in other areas compared to Port Curtis

The prevalence of lesions in Fitzroy River and Jacobs Well sampled in March/April 2000 compared to the total prevalence in Port Curtis and Ayr for 98/00 is shown in Figure 3.8. In comparing the total prevalence of disease for males and females in Port Curtis in March 2000 to that of Fitzroy and Jacobs Well for the same period, there was a significant difference between sites. There was a higher prevalence of disease at Gladstone and Fitzroy and a lower prevalence of disease at Jacobs Well ($?^2 = 10.2476$, df = 2, $0.001 < p < 0.01$). No diseased crabs were reported from Ayr.

Figure 3.8. The prevalence of rust spot shell lesion in adult mud crabs in Port Curtis in 98/00 compared to other areas sampled.



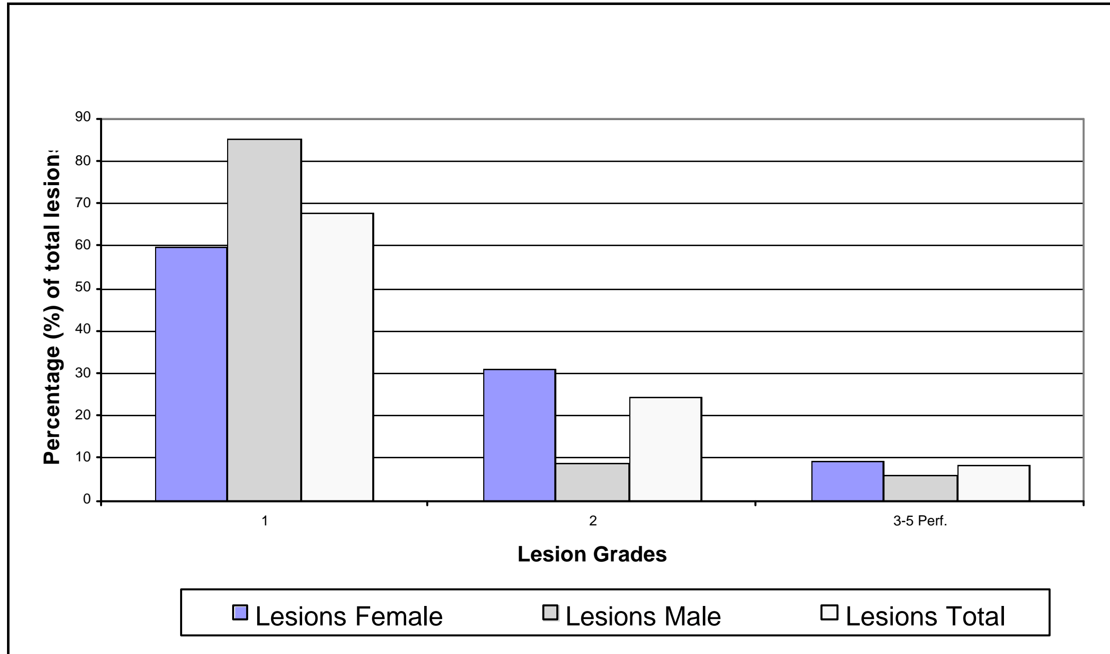
3.2.3 Grades of lesions

The bilateral symmetry of lesions on the dorsal carapace increased from 55% in 98/99 to 62.5% in 99/00. Although this increase was not significant at ?^{2} = 3.3983, df = 1, p = 0.0653, it was suggestive of a trend to more symmetry in lesions in 99/00. The results of lesion grading for 98/00 are presented in Table 3.2 and the percentages in Table 3.3. In both years females had a lower frequency of grade one lesions (lesions < $5mm^2$) than males and a higher frequency of grade two lesions (> or equal to $5mm^2$, but often these lesions were between 30mm and 60 mm in length). There was little difference between the sexes in the frequency of grade three to five lesions (perforated). (98/99 – ?^{2} = 10.7497, df = 1, p = 0.0046. 99/00 - ?^{2} = 34.6124, df = 1, $p < 0.0001$).

Table 3.2 The Grading of each rust spot lesions in male and female mud crabs and genders combined from Port Curtis in 98/00. Total lesions are shown in the bottom row.

GRADE	FEMALE	MALE	TOTAL
1	233	153	386
2	122	16	138
3-5 PERFORATED	36	11	47
TOTAL LESIONS	391	180	571

Table 3.3 Grades of rust spot lesions expressed as a percentage in male and female and gender combined mud crabs from Port Curtis in 98/00.



3.2.4 *Areas of the carapace*

The areas of the carapace to which lesions were allocated are depicted in Figure 3.5. As the frequency of lesions in area 8 was very low, this category was omitted from statistical analyses. There was a gender difference in the areas of the shell affected by lesions in both 98/99 and 99/00, with females having proportionally more lesions in areas two and five and less lesions in area seven than males. Lesion distribution for 98/99 for both female and male mud crabs is presented in Figure 3.9 and 3.10 respectively ($?^2 = 53.8538$, df = 6, $p < 0.0001$).

Figure 3.9 Lesion distribution on the carapace of female mud crabs in 98/99

Figure 3.10 Lesion distribution on the carapace of male mud crabs in 98/99.

3.2.5 Lesion frequency

The number of lesions per crab as a percentage of the total number of diseased crabs for 98/99 is demonstrated in Figure 3.11. Two lesions affected the majority of crabs and as mentioned previously, 55% of lesions were bilaterally symmetrical.

Figure 3.11. Number of lesions, (male and female combined) per crab, as a percentage of total diseased crabs in 98/99.

3.3 DISCUSSION

3.3.1 Lesion prevalence

The prevalence of rust spot shell lesions in mud crabs sampled in 2001 was significantly lower than those sampled in 98/99 and indicates a possible trend of decreasing disease prevalence in Port Curtis. The decrease in prevalence was not, however, significantly different in 2001 compared to 99/00. As the disease grading system was developed and refined over the 98/99 sampling period, it is possible that the prevalence in this year may have been slightly exaggerated due to the inclusion of wounds/other lesions as rust spot lesions, which were not included in successive years. Observations drawn from the examination of dried diseased crab carapaces that were collected in 1996 and stored, however, suggest that the prevalence was much higher in that year around when the syndrome was first noticed. Although it would appear that the prevalence of rust spot lesions in Port Curtis could be decreasing, interpretation of results should be treated with caution. Further sampling should be repeated in future years to establish any trends.

It is not surprising that rust spot lesions occur in mud crabs from other areas, as rust spot lesions are a symptom of perhaps multifactorial causes. In a sample of 29 juvenile mud crabs from an aquaculture centre, 18 had what appeared to be rust spot lesions. Subsequent histology determined that these lesions were most likely caused by a bacterial infection. In fact any damage to epidermal cells underlying the carapace will cause a rust coloured change to the carapace above. This effect was demonstrated when the epidermal cells above the gill chamber in a number of juvenile mud crabs was removed by scraping with a hypodermic needle. Within 24 hours a rust spot had developed on the overlying carapace. A similar effect was observed in 14 of 27 mud crabs obtained from Darwin, N.T. Lesions in this case were most likely due to mishandling and the prolonged time the crabs were kept out of the water. Any disruption to the cytoplasmic extensions of epidermal cells into the carapace, are a potential cause of rust spot lesions.

The prevalence of classic shell disease usually related to mechanical damage in most inshore crustacean populations is thought to be at a background level of less than 5% (Sindermann1989b). The results pose the question of why there is an increased prevalence of lesions in Port Curtis crabs compared to crabs from other areas. The similarly high prevalence of lesions in Fitzroy crabs may be because the individuals from these two locations belong to the same population, between which there is considerable migration. Hyland et al. (1984) determined that apart from an offshore spawning migration by females, there was very little free ranging type movement of mud crabs once post larval recruitment into an area had occurred. Furthermore, the mean female movement (6.6km) was significantly more than that of the males (3.7km) of the Moreton Bay mud crabs they studied. Some female crabs however, were recorded moving up to 65km. The average distance between sites in our study was less than 5km. As there was no intra-site difference in the prevalence of lesions within Port Curtis or the Fitzroy River, it is possible that the Fitzroy/Port Curtis mud crabs belong to one large population and that mixing of crabs from the two areas occurs via The Narrows, a mangrove habitat adjoining the two water bodies. Similar metal burdens have been found in female crabs from both areas, also suggesting that both groups of crabs belong to a common population.

Other researchers have reported gender differences in the prevalence of shell disease in crabs (Baross et al. 1978, Comely and Ansell 1989, Sawyer 1991, Guerin 1996). However, only Baross et al. (1978) have suggested a reason, namely that the increased prevalence in *Chionoecetes tanneri* adult females was due partly to females ceasing to moult after their pubertal moult. In contrast, however, the pubertal moult in the female mud crab is not necessarily the terminal moult (Heasman 1980). The change in gender prevalence of the disease from 98/99 to 99/00 cannot be explained at this stage, but its significance might become more apparent with repeated samplings. The higher prevalence of lesions in the warmer months could possibly be related to the increase in moulting activity that also occurs during this time, as lesions are known to form in the post moult period.

None of the wild caught juveniles examined had rust spot lesions. A number of factors apart from sexual maturity separate the adult mud crab from the juvenile mud crab including diet and habitat. Hill et al. (1982) reported that juveniles tended to reside in the mangrove zone, whereas adults were caught mainly in the deeper sub tidal area. Sub adults however, migrated into the intertidal zone to feed at high tide but retreated to sub tidal waters at low tide. The different habitats and foraging requirements occupied by each age group would suggest a disparate manner of existence and perhaps therefore different susceptibilities to disease agents. Larger adult and therefore older crabs will also have had longer exposure to the environment and various causal agents and this could be one explanation for the higher prevalence of shell disease in adults compared to juveniles.

3.3.2 Lesion severity

The high symmetry of lesions is not surprising, as histology has shown that lesions are predominantly associated with carapace muscle attachments, which are bilaterally symmetrical. Results show that the majority of lesions in females are larger than males and are located in a different area of the carapace to males. Most of the lesions in males are less than 5mm in diameter and occur in area 7. It is our opinion that most of the lesions in this area in both male and female crabs are possibly caused by natural trauma (i.e. dorsoventral compression due to a burrowing lifestyle) to the carapace epidermal cells due to impaction of the chitinous internal gill partition on the cells in this area. If area 7 lesions are disregarded very few lesions remain in male crabs. In contrast, the predominant areas affected by lesions in female crabs are areas 5 and 2. These areas coincide with underlying ovarian tissue and in some cases numerous rust spots coalesce to form a “T” zone on the carapace of female crabs, mirroring the outline of ovarian tissue. As lesions are restricted only to adult crabs, it is possible that there is a relationship between occurrence of lesions in this area and the onset of active ovaries brought on by the pubertal moult.

Non-perforated rust spots become indistinguishable once the crab is cooked. Lesions are also restricted to the shell, which is discarded in most cases prior to consumption. Perforated lesions, which are likely to downgrade saleability of mud crabs, accounted for less than 10% of affected crabs in 98-00 and therefore less than 2% of the total mud crab population in Port Curtis. As male crabs are the only gender marketed commercially in Queensland, it is therefore unlikely that marketability has been affected by the presence of rust spot lesions.

Conclusion

Over 3000 mud crabs from a number of locations in Queensland were examined for the presence of rust spot lesions between 1998 and 2001. A lesion grading system was designed to assist in accurately documenting the area of the shell affected and the severity of lesions. Although there were some gender differences in the prevalence of shell lesions in some years these were not consistent. The areas of the shell affected by lesions was, however, significantly different in males compared to females. Rust spot shell lesions did not affect juvenile mud crabs from Port Curtis. Although rust spot shell lesions were present in some other locations sampled outside Port Curtis, the prevalence was significantly lower than in Port Curtis. The prevalence of disease was, however, similar in crabs sampled from the adjacent Fitzroy River. There also appears to be a trend of decreasing disease prevalence in Port Curtis. Future sampling, however, would be required to confirm this trend. As less than 1% of the crabs marketed or consumed from Port Curtis have perforated lesions, it is unlikely that marketability has been affected by the presence of rust spot lesions.

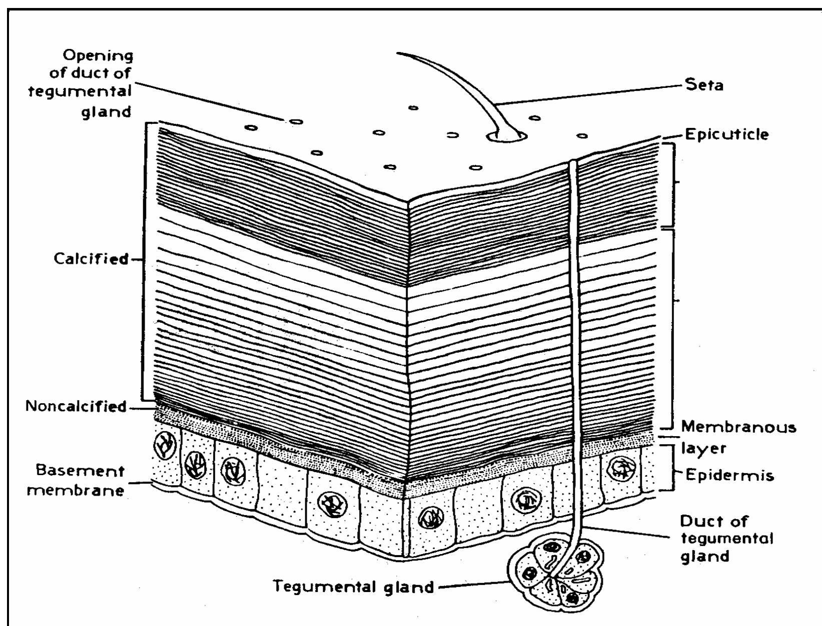
CHAPTER 4. Shell Lesion Pathology

Objective: *Define the histological stages of the lesion by developing a pathological sequence of events.*

4.1 INTRODUCTION

The cuticle of normal intermoult crabs consists of an outer epicuticle, an exocuticle, an endocuticle and an epidermis (Johnson 1980) depicted in (figure 4.1). When the crab moults, the new shell is soft and consists of an epicuticle, an as yet uncalcified exocuticle and an epidermis. During the post moult period synthesis of the endocuticle and mineralisation of all calcified layers occurs. In previous reports of shell disease, the lesions frequently develop after a breach in the epicuticle has occurred and then progress to erosion or full cuticular ulceration (Sindermann 1989b) (figure 4.2). Damage to the epicuticle, a shellac-like cement layer (Smolowitz et al. 1992), allows access by chitinolytic and lipolytic pathogens that are able to digest the chitin based exocuticle layer and in severe cases the endocuticle also.

Figure 4.1 A generalized view of the crustacean cuticle.



Adapted from Stevenson 1985

Figure 4.2 *Homarus americanus.* Classic shell lesion in the American lobster. There is erosion into the exocuticle (A), thickened membranous layer (B), mildly hypertrophic epithelium (C) and a focus of inflammatory cell accumulation (D). The calcified endocuticle remains intact (arrow).

Adapted from Smolowitz et al. 1992

In contrast, we describe a shell disease of mud crabs in which the initial lesion appears in the endocuticle while the outer exocuticle and epicuticle remain intact.

4.2 METHODS

4.2.1 Sampling and histopathology

Diseased mud crabs were captured, examined and graded as described in Chapter 3.1.1. Sixty crabs with various grades of rust spot lesions were selected for pathology. Although animal ethics approval is not required at present for invertebrates, the crabs were always handled in a humane manner and were sedated in a cold room at 6?C for approximately one hour prior to necropsy. Samples of the shell lesions, eyestalks, heart, gills, hepatopancreas, haematopoietic tissue, stomach, oesophagus, midgut, cerebral and thoracic ganglia, antennal gland, gonad, posterior midgut caecum, midgut ampulla and skeletal muscle were placed in fixative. The calcified tissues were placed in Davidson's solution and non-calcified tissues were placed in 10% formalin/seawater. The fixed and decalcified tissues were cut, blocked and stained with haematoxylin and eosin according to standard procedures. To establish whether the carapace lesions originated internally or externally, 5? m serial sections of eight non-perforated lesions (including small, medium and large lesions) were cut and every 15th section examined histologically. The tissues from a sample of 30 mud crabs without rust spot lesions and collected from throughout northern Australia, were used as a reference. Special stains for bacteria and fungi were performed on selected specimens.

4.3 RESULTS

4.3.1 Histopathology of a typical lesion

A majority of the following features was seen in each rust spot lesion. An elongated cavity, parallel to the surface of the cuticle was present in the outer endocuticle adjacent to the exocuticle (figures 4.3, 4.4). This cavity usually had a darkened edge and contained a pale-staining amorphous material. Within this cavity, the remains of adhesive epithelium (Johnson 1980) that facilitate attachment of the adjacent muscle bundle were occasionally present (figure 4.5). There were no foci of either bacteria or fungi. Proximal to the endocuticular cavity, the laminated endocuticle was usually indented (folded inwards) towards the muscle to which it had formerly attached. The muscle fibres adjacent to the attachment were replaced by fibrous connective tissue for a variable length from the point of attachment to the carapace. This fibrous tissue often contained foci of inflammatory cells. Islands of endocuticle-like material were often found within this connective tissue (figure 4.6).

In addition to the above characteristics of a rust spot lesion, those in area 7 had some additional features (figure 4.7). The calcified bilateral internal cuticular partition, which separates the gill and heart chambers, was often damaged adjacent to its association with the internal surface of the dorsal carapace. The epidermis and adjacent membranous layer of the overlying carapace were often missing or had separated from the ventral surface of the carapace. The adjacent hypodermal tissue was frequently inflamed. An elongated cavity parallel to the surface of the carapace was frequently present in the endocuticle. The epidermal cells adjacent to the lesion were often hypertrophic. From an examination of a series of sections from 8 lesions from 4 crabs, there was no evidence that the rust spot lesions had originated from the exterior via an erosion of the epi- and exocuticular layers of the carapace.

4.3.2 Histopathology of internal organs

Of the 60 crabs with rust spot lesions chosen for histopathological examination, small granulomas or focal inflammation were seen in the following tissues (numbers of affected crabs in brackets): eyes (3), heart (19), gills (11), hepatopancreas (3), stomach (1), midgut/rectum (5), posterior caecum (2), antennal gland (1) and urinary bladder (2). Sloughed cells were seen in the lumen of the antennal gland (1); Helminth parasites (intermediate stage of a tapeworm/fluke) were present in the connective tissue adjacent to the thoracic ganglion (11); baculovirus-like inclusions were present in the hepatopancreas (7); amorphous material was seen in the lumen of the antennal gland (19) and in the lumen of the urinary bladder (1). The reference group of 30 crabs had similar numbers of small granulomas, focal inflammation, baculovirus-like inclusions, helminth parasite infestations and amorphous material in the lumen of the tubules of the antennal gland, to those of the rust spot crabs.

Figure 4.3 *Scylla serrata.* A mild rust spot carapace lesion (area 2). There is a small cavity (CV) in the upper endocuticle (EN). Scale bar 126 ? m.

Figure 4.4 *Scylla serrata.* A severe rust spot carapace lesion (area 2). There is a large cavity (CV) in the upper endocuticle; the lower endocuticle is indented (IN); fibrous connective tissue (FB); muscle tissue (MS). Scale bar = 126 ? m

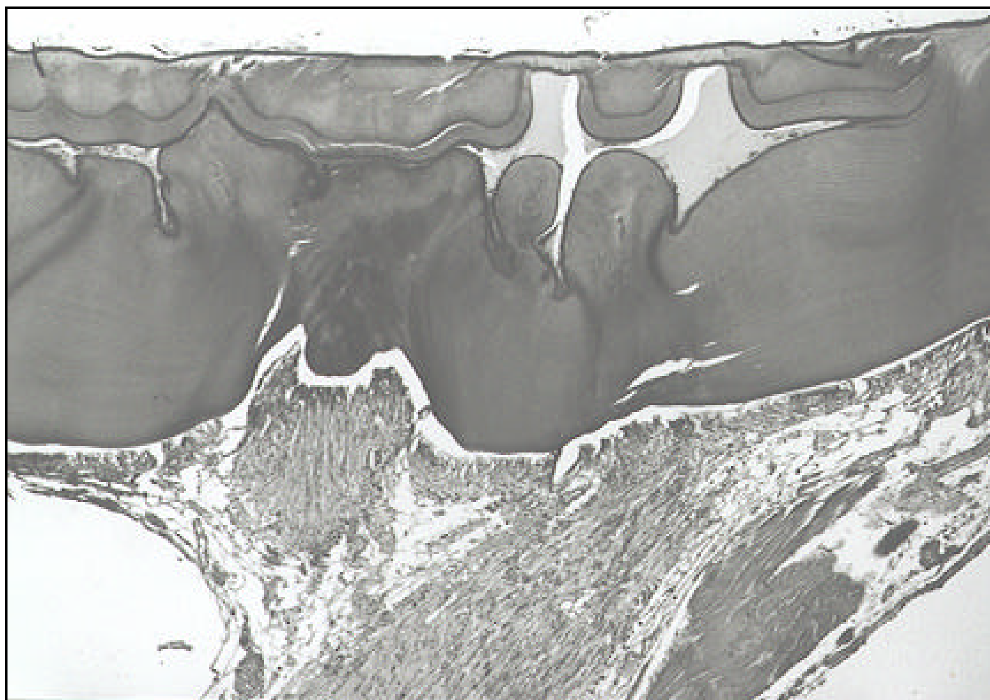


Figure 4.5 *Scylla serrata.* A severe rust spot carapace lesion to show the remaining muscle adhesive epithelium (AD) present in the cavity (CV) in the upper endocuticle (EN). Scale bar 126 ? m

Figure 4.6 *Scylla serrata.* An island of endocuticle (IS) is present in the fibrous connective tissue (FB) between the endocuticle (EN) and the attached muscle (MS) of a severe rust spot carapace lesion (area 2). Scale bar = 126? m.

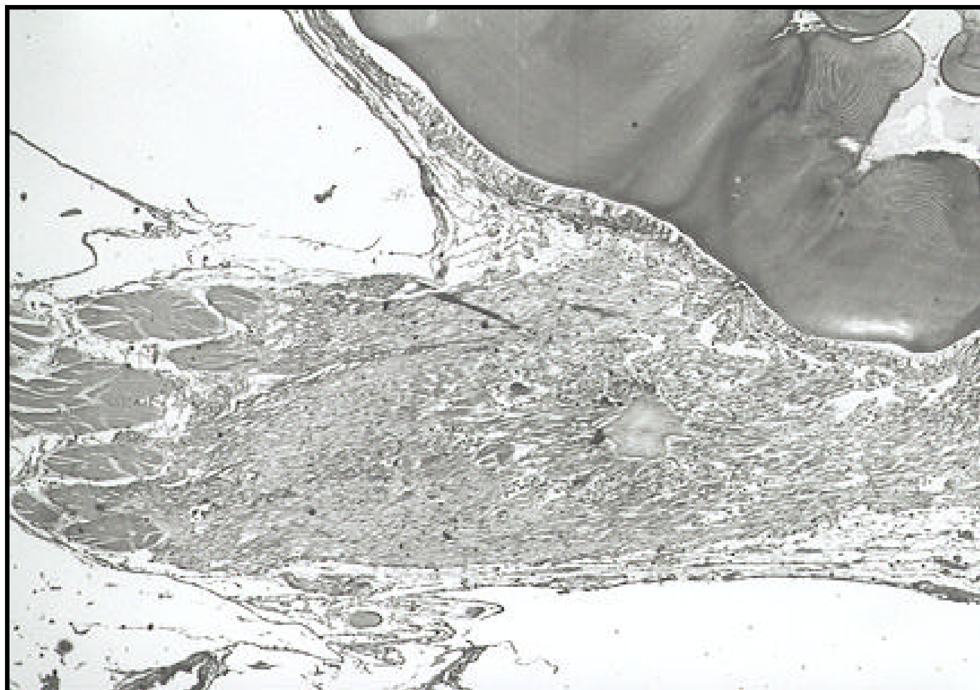
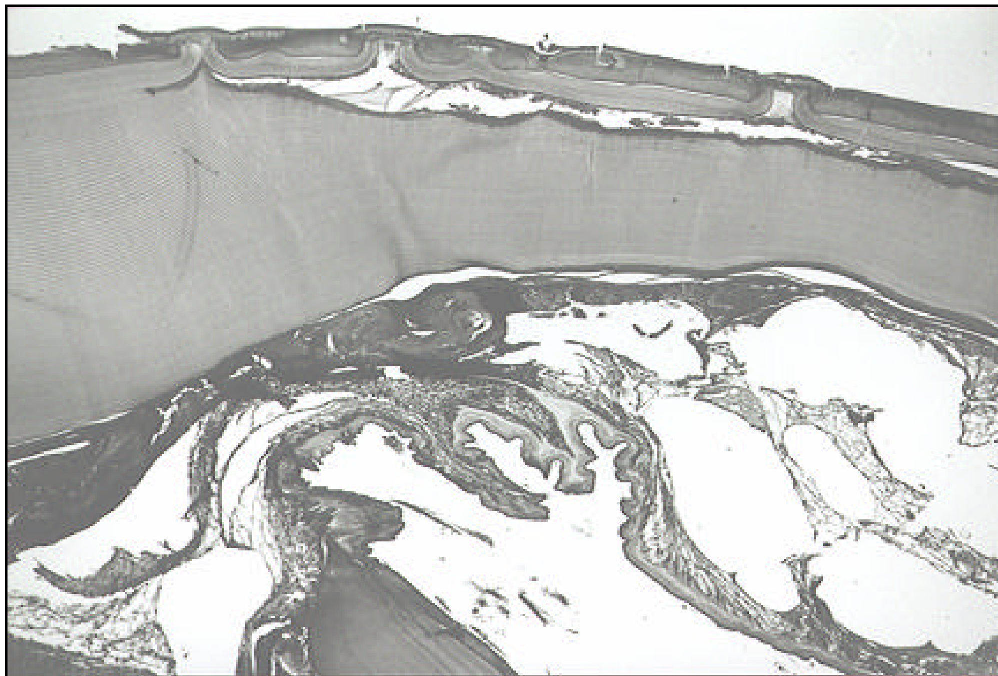


Figure 4.7 *Scylla serrata.* A moderately sized cavity (CV) in the endocuticle (EN) of the carapace (area 7) above damaged epidermis (EP) and an internal cuticular partition (PT). Scale bar = 212?m.



4.4 DISCUSSION

The pathology of non-perforated rust spot lesions in mud crabs harvested from Gladstone harbour is restricted to the endocuticle and the adjacent muscle attachment. It is distinctly different to previously described shell disease (Rosen 1967, Baross et al. 1978, Morado et al 1988, Comely and Ansell 1989, Smolowitz et al. 1992, Noga et al 2000) in other crustacea where the initial action is an erosion of the exocuticle resulting in eventual exposure of the endocuticle to the external environment. Although some have suggested a possible systemic pathway for the initiation of shell disease (Comely and Ansell 1989, Smolowitz et al. 1992), a widely held view is that crustacean shell disease is caused by ubiquitous, chitinolytic, microorganisms acting alone or in concert (Sindermann 1989a). Others have suggested that an impaired host defence is an important contributing cause (Prince et al. 1993, Noga et al.1996b). Sequential sections of non-perforated rust spot lesions of mud crabs, however, have demonstrated that it is unlikely that chitinoclastic pathogens had gained entry into the endocuticle via erosions of the epicuticle and exocuticle. Cuticular pores have also been suggested as portals of entry for bacteria and other microorganisms in lobsters (*Homarus americanus*) (Prince et al. 1993). However, the lack of any foci of bacteria or fungi in our non-perforated rust spot lesions suggests a different aetiology. Furthermore, from an examination of the internal organs, the lack of any evidence of an infectious or parasitic agent being associated with the lesions suggests that the likely cause is non-infectious. Since the endocuticular layer is formed in the post moult phase of the moult cycle (Travis 1957), it would appear that the lesions occur due to a defect in the formation of this endocuticular layer rather than as a result of pathogenic cuticular erosion.

We suggest the following hypothesis to explain the pathogenesis of rust spot lesions. At ecdysis, approximately 90% of the whole body calcium is lost with the exuviae (Scott-Fordsmand and Depledge 1997). After moulting, when the endocuticle is being laid down, a rapid active uptake of calcium occurs from the environment (Greenaway 1985) allowing cuticular calcification to take place. Interference with this process of calcification could prevent the uncalcified endocuticle from attaining the necessary strength to support the forces exerted by the muscle insertions. Premature contraction of these muscles would cause either (1) the non-calcified endocuticle to rupture between the laminations which run parallel to the surface of the carapace or (2) the adhesive epithelium of the muscle attachment to tear away from the partially calcified endocuticle. These processes could produce a cavity in the endocuticle or one beneath the endocuticle, with some of the adhesive epithelial cells of the muscle attachment sometimes remaining in the latter cases. The dislocated epidermis would continue to produce more layers of endocuticle proximal to the cavity. Local repair mechanisms including inflammation and fibrous tissue replacement would occur in an attempt to heal the muscle adjacent to the endocuticle. Islands of endocuticle may be formed in this fibrous tissue from segments of torn and dislocated epidermis that have folded in on them selves and have continued to produce endocuticle. Once a cavity has formed in the endocuticle, or the epidermis has been pulled from the endocuticular surface, the pore canals containing cytoplasmic extensions of the epidermis, which extend into the overlying cuticular layers (Roer and Dilliman 1984) are severed. The cuticle exterior to the cavity 'dies' and assumes a rust-coloured appearance.

In conclusion, our findings indicate that rust spot lesions are part of a new type of shell disease with a unique histology compared to previously reported cases of shell disease. Although the epidemiology is still not completely understood, the hypothesis incorporating the possibility that inhibition of calcium uptake is a contributing factor to the development of lesions, is discussed in Chapter 8. A paper has been published on the pathology of the unique histological lesions and a reprint is included in Appendix 3.

Conclusion

Over 60 mud crabs with rust spot lesions were chosen for histopathological examination and the results compared to a reference group of 30 non-diseased mud crabs. There was no evidence of an infectious or parasitic agent of significance being associated with any internal organ of the rust spot crabs. The pathology of the rust spot lesions is restricted to the endocuticle layer (internal shell layer) and adjacent muscle attachment. As the endocuticular layer is formed in the post moult phase i.e. after the crab has moulted, it appears that the lesions are caused by a defect in the manufacturing of this layer, while the crab is in the process of calcifying its shell. This is in contrast to previously reported shell disease in other crustaceans, where the pathology is an external erosion of the shell, which may be caused by pathogenic organisms.

CHAPTER 5. Transmission Experiments

Objective 3: *If an infectious agent is isolated/identified, determine its ability to cross infect to other species of crustaceans.*

5.1 METHODS

5.1.1 Inoculation trial: sand crabs

Aim: *To determine if the tissues of Gladstone crabs with rust spot carapace lesions could transmit this disease to sand crabs* (Portunus pelagicus).

Groups of juvenile sand crabs *P. pelagicus* (4 to 6 cm shell width) were inoculated into the haemocoele with 0.2 ml of a homogenate of either epidermis, hepatopancreas, gill or normal saline. The material for each inoculum was prepared from tissues from two Gladstone mud crabs with rust spot lesions, was passed through a 0.2?m filter. Each crab was kept in a separate glass aquarium (minimum volume 20 litres) with aeration and a biological filtering system. The crabs were fed a diet of squid and mullet every 3 days. All wastes were removed daily. Water changes were made between every second to seventh day when the nitrite concentration reached 1 ppm. Four weeks after inoculation, the crabs were examined grossly, necropsied and histopathology done. Tissues examined included eyes, epidermis, heart, gills, hepatopancreas, stomach, caeca, central nervous system, antennal gland, hindgut, skeletal muscle and haematopoietic tissue.

5.1.2 Inoculation trial: marine prawns

Aim: *To determine if the tissues of Gladstone crabs with rust spot carapace lesions could transmit this disease to marine prawns* (Penaeus monodon).

30 marine prawns *P. monodon* were divided equally into five groups and each inoculated intramuscularly with 0.1 ml of a homogenate of epidermis, hepatopancreas, gill or normal saline (controls). Pooled tissues for each inoculum obtained from two Gladstone mud crabs with rust spot carapace lesions, were passed through a 0.2?m filter. Each group of 6 prawns was kept in a glass aquarium with a minimum volume of 50 litres with its own biological filter and aeration system. The prawns were fed prawn pellets twice a day and wastes were removed daily. Water quality was monitored regularly and the water was changed if the nitrite concentration reached 1ppm. Four weeks after inoculation, the prawns were examined grossly, fixed in Davidson's solution and processed for histopathology. Tissues examined included heart, gills, antennal gland, central nervous system, eye, hepatopancreas, midgut, caeca, stomach, epidermis, lymphoid organ and skeletal muscle.

5.1.3 *Inoculation trial: mud crabs*

Aim: *To determine if the tissues of Gladstone crabs with rust spot carapace lesions could transmit this disease to mud crabs* (Scylla serrata).

Four groups of eight juvenile mud crabs *S. serrata* (20 to 30 mm shell width) were inoculated into the haemocoele with 0.1 ml of a homogenate which had been passed through a 0.2? m filter, prepared from tissues from two Gladstone mud crabs with rust spot lesions. Each treatment group was inoculated either with epidermis, hepatopancreas, gill or normal saline (controls). Each crab was kept in a separate glass aquarium (minimum volume 20 litres) with aeration and a biological filtering system. All crabs were fed a diet of squid and mullet every three days and all wastes were removed daily. Water changes were made every two to seven days or whenever the nitrite concentration reached 1 ppm. Four weeks after inoculation, the crabs were examined grossly, necropsied and histopathology performed. Tissues examined included eyes, epidermis, heart, gills, hepatopancreas, stomach, caeca, central nervous system, antennal gland, hindgut, skeletal muscle and haematopoietic tissue.

5.1.4 *Water transmission trial: adult mud crabs*

Aim: *To determine if rust spot lesions can be induced in unaffected adult mud crabs (Scylla serrata) by exposure to Gladstone water.*

Adult intermoult crabs (8 males and 2 females, carapace width 145mm to 180mm) from a pristine area (Eurimbula National Park-Agnes Water) showing no gross carapace lesions, were selected for the trial. Crabs were divided equally into two treatment groups.

1. Gladstone Harbour water, obtained on an ebb tide from an area where diseased crabs have been regularly caught (treatment).

2. Combination ocean water from outside the harbour and artificial seawater (control).

Crabs were kept individually in 10 randomly placed separate aquaria, each with its own biological filtering system. Prior to the experiment each individual was measured, weighed and blood samples were taken. Crabs were fed daily on a mixed diet of prawns, Moreton Bay bug heads, squid and white pilchards. Solid wastes were removed daily. The water temperature, nitrite, ammonia, pH, salinity and dissolved oxygen were recorded weekly and the water in each aquarium partially changed if required. After five weeks crabs were examined for lesions, necropsied and tissue and blood samples preserved for later use.

5.1.5 Water transmission trial: juvenile mud crabs

Aim: *To determine if rust spot lesions can be induced in unaffected juvenile mud crabs (Scylla serrata) by exposure to Gladstone water.*

As histology had demonstrated that rust spot lesions form in the post moult phase, the trial was repeated with juvenile mud crabs, which have a short intermoult period (5 - 14 days). These crabs were likely to moult several times during the 12-week experiment, compared to adults in which the time between moults spans several months to over a year. Juvenile mud crabs (48) were obtained from Darwin Aquaculture Centre (sex undetermined, carapace width 11-18mm) and were randomly allocated to the two treatment groups:

1. Gladstone harbour water, obtained on an ebb tide from an area where rust spot crabs have been regularly caught (8 replicate tanks).
2. Control water, a combination of ocean water from outside the harbour and artificial seawater (8 replicate tanks).

Twenty-four crabs, subdivided into eight groups of three, were used in each treatment. Each replicate consisted of three crabs individually caged in plastic containers suspended within an aquarium with its own biological filtering system (min. vol. 60 l)(Figure 5.1) in a temperature-controlled room. Crabs were fed prawns, white pilchards or squid daily and wastes were removed daily. When crabs reached a carapace depth of 15mm their plastic container was exchanged for a larger mesh sided cage suspended in the same aquarium to improve aeration. Crabs were examined weekly under a dissecting microscope for the presence of gross shell lesions and also weighed. If the crab had moulted since the last examination, the new carapace width and depth was recorded. The temperature, nitrite, ammonia, pH, salinity and dissolved oxygen were recorded weekly and the water in each aquarium was partially changed if required. After 12 weeks all remaining crabs were examined for evidence of rust spot shell lesions.

Figure 5.1 Juvenile mud crab used in transmission trials (A). Plastic containers (B) and mesh sided containers (C) used to house mud crabs, floating in aerated aquarium.

5.2 RESULTS

5.2.1 Inoculation trial: sand crabs

One of the control crabs died from an aeration failure and one of the epidermis group crabs died of undetermined causes. No shell lesions were recorded and no significant lesions were seen in any of the tissues.

5.2.2 Inoculation trial: marine prawns

The number of prawns that died or were cannibalised in each treatment group was small (Table 5.1). No gross shell lesions were recorded and no significant histopathological lesions were found.

Table 5.1 Number of surviving prawns in a four-week inoculation trial involving young adult marine prawns *Penaeus monodon* inoculated with tissues from Gladstone rust spot mud crabs.

Inoculated Material	Tank No	No. of Prawns Inoculated	No. of Prawns Necropsied After 4 Weeks	Total Surviving Prawns/ Group
Control (normal saline)	36	6	5	
	39	6	5	
	43	6	6	28
	46	6	6	
	50	6	6	
Epidermis	38	6	6	
	40	6	6	
	42	6	5	27
	52	6	5	
	49	6	5	
Gill	33	6	6	
	37	6	6	
	47	6	6	30
	44	6	6	
	51	6	6	
Hepatopancreas	34	6	4	
	35	6	5	
	41	6	5	25
	45	6	5	
	48	6	6	

5.2.3 Inoculation trial: mud crabs

None of the 32 mud crabs exhibited rust spot shell lesions and none died. No other significant lesion was seen in any of the tissues.

5.2.4 Water transmission trial: adult mud crabs

One control crab died of undetermined causes after 24hrs, another control crab died while escaping and a third control crab died after filter failure, four weeks into the experiment. No gross rust spot shell lesions were observed in either the treatment or control groups.

5.2.5 Water transmission trial: juvenile mud crabs

Three crabs died when aquarium aeration failed, three died of undetermined causes within the first few days of the experiment and one died of moult death syndrome (MDS). There was no evidence of rust spot lesions in crabs from either the treatment or the control group.

5.3 DISCUSSION

From our experiments and our pathological examination there is no evidence to suggest that mud crabs with rust spot disease contain a virus or virus-like organism capable of transmitting this disease to sand crabs, marine prawns, juvenile or adult mud crabs. Failure to reproduce rust spot lesions in unaffected mud crabs and other crustacean species by transmission experiments also suggests a non-viral cause of this disease. From the examination of non-perforated lesions and internal organs of diseased crabs, the lack of any evidence of an infectious (i.e. bacterial or fungal) or parasitic agent would tend to suggest that the likely cause is non-infectious.

Shell disease in other crustaceans has been associated with many infectious agents (Sindermann 1989a), with the majority of cases being linked to various bacteria, primarily the genera *Vibrio* and *Pseudomonas* (Cook and Lofton 1973, Borkowski and Bullis 1989, Prince et al 1993). Some authors have had some limited success in the experimental transmission of bacterial (Malloy 1978) and fungal (Alderman 1981) shell disease through inoculation trials. Demonstration of a shell "infection," however, requires that one identify pathogens that are unique to shell disease or at least are in greater numbers in diseased animals (Noga 1991). As the normal crustacean carapace is heavily colonized with ubiquitous microbes (Baross et al 1978, Noga et al 2000) and there has been little quantitative work to compare relative numbers of pathogens present in diseased compared to non-diseased animals (Noga 1991), it is unlikely that the cause of classic shell disease in other crustaceans is solely microbiological.

The unique histology of rust spot shell disease strongly suggests that the prime cause is not microbiological (Chapter 4.) Therefore it is not surprising that rust spot shell disease was not able to be reproduced in these transmission trials. It is likely, however, that in perforated shell lesions, pathogenic microorganisms may become secondary invaders. In conclusion it appears that there is negligible risk of cross infection from crabs with rust spot shell lesions to other crustacean species.

Conclusion

Cross infection experiments were conducted in an attempt to transmit rust spot shell disease to other mud crabs, sand crabs and prawns. In water transmission trials, adult and juvenile mud crabs were exposed to Gladstone Harbour water. In inoculation trials, processed tissue from diseased mud crabs was injected into juvenile mud crabs, sand crabs and prawns. None of the treatment groups developed rust spot lesions and after subsequent pathology, no significant lesions were seen in any of the tissues examined. It appears that rust spot lesions do not contain a virus or virus-like organism capable of transmitting the disease to other mud crabs or crustaceans. It is probable that the cause of rust spot shell lesions is non-infectious.

CHAPTER 6. Moulting Experiments

Objective 1: *Determine if affected crabs are able to moult and therefore mate successfully by experimentally observing diseased male and female crabs. Determine if the crab can shed the ulcerations, during a moult. Is there a healing stage?*

6.1 INTRODUCTION

Rust spot carapace lesions whether non-perforated or perforated, have the potential to inhibit the moult process by causing adhesions, which could prevent the exuviae from being shed. In classic shell disease, adhesions have been known to prevent ecdysis (Sindermann 1989a). Large calcareous outcrops are often present on the ventral surface of the carapace underlying most non-perforated rust spots. Once ulceration has occurred, the epithelium is destroyed/lost and the potential for production of the new carapace doubtful. As moulting is required for mud crabs to grow and also to reproduce (mating only occurs while the female is in the soft post moult phase), the ability of diseased crabs to shed lesions during a moult was investigated.

6.2 METHODS

Over the course of the project eight diseased adult mud crabs from Port Curtis, with various grades of lesions were kept in individual 60 l aquaria, each with its own biological filtering system and fed on prawns, pilchards and bivalves. Crabs were sexed and weighed and lesions recorded both photographically and diagrammatically on commencement of the experiment using the previously developed grading system (Chapter 3). Crabs that moulted were kept and observed over the post moult period and changes in previous lesions recorded over time. Observations were also made for the appearance of new lesions. A number of observations were also recorded from 29 pond-reared juveniles obtained from an aquaculture centre, which were kept in similar conditions and observed over an eight-week period.

6.3 RESULTS

The results of the moulting trials are presented on a case-by-case basis.

6.3.1 Case 1

Case 1 was a male crab, 115mm carapace width with three perforated lesions (figure 6.1). Case 1 moulted 9 weeks after being placed in captivity and was seen to heal all three lesions to some extent (figure 6.2). The new carapace width measured within 24hrs was 130mm. In the proceeding five days post moult, however, case 1 developed a number of new rust spot lesions (figure 6.3).

6.3.2 Case 2

Case 2 was a male approximately 150mm carapace width, with three lesions, one of which was quite severe (figure 6.4). Case 2 was also missing eight limbs, but moulted eleven weeks into captivity, healing old lesions and regenerating new limbs (figure 6.5). New lesions, however, developed post moult.

3

2

1

Figure 6.1 Case 1, 29/1/99 prior to moulting showing three separate perforated lesions (numbered).
Lesion description Case 1

Lesion No.	Area of shell	Grade	Comment
1	3	3	Large area of exposed endocuticle
2	3	5	Perforated rust spot
3	1	3	Ulcerated, deformed orbit

3

4

2

1

Figure 6.2 Case 1 8/4/99, one day post moult showing healing of all three lesions and development of a new lesion (4).

Lesion No.	Area of shell	Grade	Comment
1	3	3	New shell reduced deficit
2	3	5	New shell almost closed deficit
3	1	3	Closure of deficit but still deformed
New			
4	6	N/A	New raised blister

1 7 8

10

9 11

Figure 6.3 Case 1, 13/4/99, five days post moult, showing development of a number of new lesions.

Lesion No.	Area of shell	Grade	Comment
1	3	3	No change since 8/4/99
2	3	5	No change since 8/4/99
3	1	3	No change since 8/4/99
New			
4	6	N/A	No change since 8/4/99
5	2	1	Rust spot
6	6	2	Rust spot
7	7	1	Rust spot
8	7	1	Rust spot
9	3	1	Rust spot
10	4	2	Rust spot
11	4	1	Rust spot

Figure 6.4 Case 2 (taken 25/1/99), premoult. The main feature is the large cuticular deficit exposing underlying necrotic gill filaments. There is a large crack in the adjacent cuticle. Note the growth of new limb buds.

Lesion No.	Area of shell	Grade	Comment
1	7	1	Rust spot
2	7	1	Rust spot
3	3	5	Large shell deficit exposing necrotic
			gills, large crack running anteriorly

Figure 6.5 Case 2 one-day post moult. Two new blisters have developed (lesion 4 and 5). The main lesion has a new edge of endocuticle (A), which has helped to reduce the cuticular deficit. The new wound edge is lined with setae, similar to that found on the edges of other parts of the exoskeleton. Some of the necrotic gills have been regenerated and the crack in the cuticle anterior to the main lesion has healed. Note the regeneration of new limbs.

Lesion No.	Area of shell	Grade	Comment
1	7	1	Now a blister
2	7	1	Now a blister
3	3	5	Crack has healed, some necrotic
			gills replaced, deficit reduced by
			a 5mm new edge of exocuticle
New			
4	7	N/A	New raised blister
5	7	N/A	New raised blister

Figure 6.6 Case 2, four weeks post moult (10/3/99). New blisters have developed into rust spots and the new edge has calcified.

Lesion No.	Area of shell	Grade	Comment
1	7	1	Rust spot
2	7	1	Rust spot
3	3	5	New edge calcified
New			
4	7	1	Rust spot
5	7	1	Rust spot

6.3.3 Case 3

Case 3, a male crab 145mm carapace width who had one severe lesion similar to case 2 (figure 6.7), began moulting 4 months into captivity but died before completely shedding its exuviae. The ulceration in the underlying newly formed soft shell, however, had healed in a similar manner to Case 2 (figure 6.8).

Figure 6.7 Case 3 showing severe ulceration on carapace exposing gill filaments. There are some signs of previous healing (arrow), possibly at a previous moult.

Lesion No.	Area of shell	Grade	Comment
1	2/3	5	Large deficit over gills similar to Case 2
			Possibly some healing in previous moult?

Figure 6.8 Case 3 (deceased) within hours of moulting showing some healing of the ulceration and a clot of congealed haemolymph within the cavity. The partially shed exuviae was manually removed.

6.3.4 Cases 4-8

The last five cases (four females and one male) were kept for observation for four to six months but did not moult during this period. All crabs had at least two lesions

ranging from Grade 1 to 5. Non-perforated lesions (Grade 1-2) on two of the four crabs had progressed in severity during the period of captivity to become perforated (Grade 3). In these lesions the exocuticle had deteriorated to expose the underlying endocuticle.

6.3.5 Pond reared juveniles

Of the 29 pond-reared juveniles examined, 62% had carapace lesions. Subsequent pathology demonstrated that although some lesions had some similarities to rust spot lesions, they were more likely to be bacterial in nature and were assumed to be due to complications arising from impoundment. Seven of the ten crabs that began moulting died of moult death syndrome. Six of these had shell lesions, however, the three crabs which successfully moulted were also diseased. Two of the these crabs developed new lesions within ten days of moulting and four crabs which were soft shelled on first examination also developed new shell lesions within the same time frame.

6.4 DISCUSSION

Some of the lesions on the crabs featured in the previous observations were of the severest grade. These crabs were caught from the wild and appear to be able to function normally in captivity, despite their afflictions. Both cases that had moulted successfully had also increased in size (carapace width) by 10-13%, which was at the lower end of the normal range of moult increment suggested by Heasman (1980), for wild caught adult crabs. Crabs were also measured within 24hrs of moulting and may not have attained an equilibrium size (usually 1-2 days) at the time of examination (Heasman 1980). Therefore, an increase in growth suggests the crabs were coping with normal bodily functions despite their diseased state.

The moult process is one of the most stressful stages in the crustacean life cycle during which many intense physiological changes occur (Skinner 1985). Two of the three moulting crabs were not only able to moult successfully but also able to regenerate new limbs. Death during the moulting process is termed “moult death syndrome” and although the cause is unknown, in juveniles it is thought to be multifactorial and strongly influenced by nutritional and environmental factors including low oxygen availability (information obtained from internet crustacean mailing list). The cause of death in Case 3 is unknown but the presence of a large amount of congealed haemolymph inside the ulceration and also exuding from the oral cavity suggests that the death may have been caused by a ruptured blood vessel or sinus. It is unknown whether death was related to the presence of the severe shell lesion. Noga et al. (2000) in their observations of Pamlico River shell disease in blue crabs suggested that it was “highly unlikely” that crabs with perforated lesions in particular, would be able to moult due to the adhesions and fibrosis associated with these lesions. Although none of the crabs they observed moulted, they did note that they were weaker and had poor survival rates compared with other crabs. The crabs with severe lesions in our experiment, however, appeared to behave no differently to the non-diseased crabs.

The epidermis is responsible for the production of new cuticle in the post moult phase. In the case where large ulcerations occur which includes loss of the epidermis, closure of the wound can only be brought about by production of new cuticle from the epidermis at the external edges of the wound. Halkrow (1988) demonstrated wound repair in four malacostracan crustaceans. He noted that after wounding, a repair

"bridge" of new cuticle formed underneath a plug of clotted haemolymph and disintegrating haemocytes, which filled the ulcer. The new repair cuticle, however, lacked an epicuticle, the protective outer waxy layer of normal cuticle, which is an important barrier to external pathogens. After a moult, however, the epidermis is able to produce a new structurally complete cuticle (containing all layers) extending out across the ulcer. With each successive moult, new cuticle is formed until eventually the new edges meet, filling in the gap.

Old lesions were shed with the old exuviae and a healed form of the lesion remained in some cases in the new cuticle. New lesions were seen to form in the post moult phase (during production of the new endocuticle) and would most likely remain until the next moult. It is not surprising that non-perforated Grade 2 lesions were seen to progress in severity over time. Histology has shown that where lesions occur there is disruption of the cytoplasmic extensions which supply the cuticle with haemolymph and nutrients and also therefore, immune factors. The cuticle in this area is also thinner and more friable due to the presence of underlying cavities that in combination with the above factors predispose the cuticle to degradation by chitinoclastic pathogens (Andersen *et al* 2000).

In conclusion we have determined that diseased mud crabs even with severe lesions are able to moult successfully and repair shell lesions. In cases where lesions are extensive, however, they may contribute to the cause of moult death syndrome. Although the crabs we observed were kept in captivity, increasing their chances of survival, we have also observed large healed scars on the carapaces of wild caught crabs. These crabs have obviously successfully moulted, possibly on more than one occasion while at the same time systematically repairing severe ulcerations.

Conclusion

A number of diseased mud crabs both adult and juvenile were observed through a moulting period. Old lesions were shed with the old exuviae and a healed form of the lesion remained in some cases. New lesions were also seen to form in the post moult phase (during production of the new endocuticle) and would most likely remain until the next moult. Although diseased mud crabs even with severe lesions are able to moult successfully and repair shell lesions, in cases where lesions are extensive, however, they may contribute to the cause of moult death syndrome.

CHAPTER 7. Metal Burdens

Objective: *Investigate metal burdens of mud crabs from Gladstone compared to other areas.*

7.1 METHODS

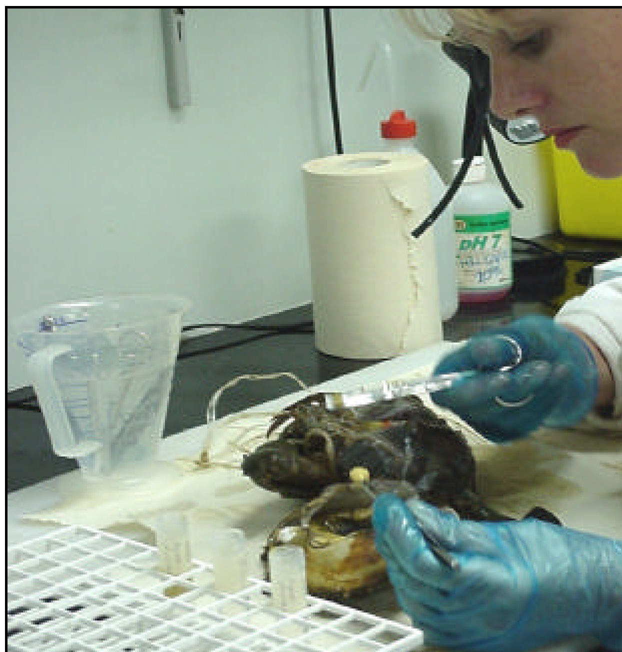
7.1.1 Collection of specimens

Crabs were obtained live from commercial fisherman and examined. Examination included sexing, measuring the carapace width (to the nearest 5mm) with a standard metric ruler and recording details of any carapace lesions. Diseased crabs were graded according to the method discussed previously (Chapter 3). The 1999 sampling focused on Gladstone non-diseased compared with diseased crabs and Gladstone crabs as a whole in comparison to crabs from a control site. Ten diseased mud crabs (mixed gender), each with at least one large, grade two (non-perforated) rust spot lesion, ten non-diseased mud crabs from Gladstone (Glad 99) and ten non-diseased male mud crabs from Ayr (Ayr 99) were selected for metal analyses.

In the 2000 sampling, a summary was made of comparisons of metal concentrations in crab hepatopancreas samples by sites (CT = Ayr/GS = Gladstone), sexes (M = male/F = female) and condition (DS = diseased/ND = non-diseased). Fifteen diseased (DS) female mud crabs from Gladstone, each with at least one large, grade two (non-perforated) rust spot lesion, were selected for toxicological analyses. Due to unavailability, no diseased male crabs were used in this comparison. Non-diseased (ND) mud crabs from Gladstone (GS) (fifteen females and fifteen males) and Ayr (CT) (fifteen females and fifteen males) were also collected for comparison. An analogy was also made between hepatopancreas metal concentrations from 10 non-diseased female crabs from Fitzroy River collected in 2000. Although no animal ethics approval is currently required for research with invertebrates, our mud crabs were always handled in a humane manner.

7.1.2 Metal analyses

Crabs were anaesthetised for dissection by chilling (–5 to -20°C) for one hour prior to euthanasia. Samples of hepatopancreas and muscle tissue (1-5g) were removed and held frozen (-20°C) for subsequent metal analyses taking care to minimize contamination (Figure 7.1). Nitric acid was added to a weighed sub sample of tissue, which was then digested by microwave in a sealed digestion vessel. After dilution with ultra pure water the sample was analysed using Inductively Coupled Plasma – Mass Spectrometer (ICP-MS)(Perkin-Elma) for a heavy metal sweep through the periodic table and concentrations reported on a wet weight basis (mg/kg).



7.1.3 Statistical treatment of results

7.1.3.1 Hepatopancreas analyses 1999

As there were several measurements (metals) for the same individual, a MANOVA (multivariate analysis of variance) was considered the appropriate analysis for difference between diseased/ non-diseased and Ayr/Gladstone. All metals that were all or mostly <0.1 were excluded from the analyses. If an overall result was significant then each metal was then examined separately in a one-way ANOVA design.

7.1.3.2 Hepatopancreas analyses 2000

Prior to analysis, any metals with all values less than detection (<0.1 mg/kg) were deleted. This left 28 metals with at least one value above detection. For any remaining values reported as less than detection, half the detection level was taken as the value to be used in analysis (i.e. for a detection level of <0.1 mg/kg, a value of 0.05 mg/kg was used). Sexes, sites and condition were kept separate to give 5 levels in a one-way ANOVA design (GS-ND-M, GS-ND-F, GS-DS-F, CT-ND-M and GS-ND-F). An *a posteriori* Tukeys HSD multiple range test was used to locate differences between levels where a significant main effect was recorded

7.1.3.3 Hepatopancreas 1999 and 2000 comparison

A summary was made of comparisons of hepatopancreas metal concentrations in mud crabs from Ayr versus Gladstone in 1999 versus 2000. For these analyses, sexes were combined at each site (Ayr & Gladstone). Due to there being insufficient data in 1999 to allow gender and lesion/non-lesion comparisons, crabs were also combined into a 'Gladstone' sample. Even though there was a larger sample size, in the majority of instances equality of variances could not be achieved using normal, square root or log10 transformed data, and therefore, non-parametric Kruskall-Wallis ANOVA was applied. This test does not allow a two-way comparison (i.e. Year x Exposure), therefore, data were coded into four levels; Ayr – 1999, Ayr 2000, Gladstone – 1999;

Gladstone – 2000. For those metals with a sign difference, means (+ 95 % confidence intervals) were plotted.

7.1.3.4 Hepatopancreas within group variation 1999 and 2000

To test whether any group had higher or lower variability in metal concentrations compared to other groups, the Coefficient of Variation (CV) being the ratio of mean concentration to the standard deviation, expressed as a percentage was calculated for each of the six groups (CT99, CT00, DS99, DS00, ND99, ND00). The CV for each metal was then used as replicates in an ANOVA approach to test for between-year and group differences (this assumes that each metal behaves the same and is equally likely to have a high/low variability in a group – which may not be true). Two-way ANOVA between years (1999 – 2000) and groups (CT, DS & ND) was performed with *a posteriori* Tukeys HSD Multiple Range test to determine between-year/group differences.

7.1.3.5 Hepatopancreas 2000 metal burdens

During statistical analysis of differences in metal concentration between CT, DS and ND crabs in 1999 and 2000, parametric tests could not be used because of inequality of variances between the six samples/groups (CT99, CT00, DS99, DS00, ND99, ND00). In plotting the data it appeared that for most metals, concentration in DS00 in particular had very high variability which, confounded efforts to establish significant differences between groups. Therefore an attempt was made to look at total metal burdens of each group and to see if a relationship existed between metal burden and shell disease. Data standardization and analysis were undertaken to compare total metal burden between the five groups of crabs:

Gladstone – Non diseased – Males (GS-ND-M) (n=15)
Gladstone – Non diseased – Females (GS-ND-F) (n=15)
Gladstone –Diseased – Females (GS-DS-F) (n=15)
Ayr – Non-diseased Males (CT-ND-M) (n=15)
Ayr – Non-diseased Females (CT-ND-F) (n=15)
Metal concentrations in crab hepatopancreas tissues from 2000 (total of 75 samples) were used to calculate total metal burden according to the following steps:

All metals for which at least one value out of the 75 samples exceeded detection (> 0.1 mg/kg) were retained for analysis – this left 28 metals with at least one value above detection. Within these metals, if any values were less than detection, half the detection level was taken as the value to be used in analysis (i.e. for a detection level of <0.1, a value of 0.05 was used).

Within each metal, the values were standardised to a scale of zero to 1 by the equation: xnew = (x-xmin)/xrange. This provides a relative indication of the burden of each metal in each sample (i.e. samples with a high concentration of a metal will have a value close to 1 and samples with a low concentration will have a value close to zero). To derive a total burden of all metals in each of the 75 samples, the sum of the individual burdens across the 28 metals was calculated. This provided a total metal burden for each of the 75 samples. Mean and 95% confidence intervals for each group of samples was calculated, and between-group differences in metal burden was tested using one-way ANOVA with the 15 samples within each group acting as replicates.

Between-group comparisons in metal burden were made in a one-way ANOVA design. Bartletts test was used to check homogeneity of variances.

7.1.3.6 Hepatopancreas 2000 multivariate analyses

An alternate statistical method to that used in metal burdens was applied to the year 2000 hepatopancreas data, in an effort to classify objects (crabs) using attributes (metal concentrations). Crabs with similar metal concentrations would ordinate together and away from those with different concentrations, therefore giving us a visual overview of differences in metal concentrations among our five groups of crabs (as above).

The CSIRO Pattern Analysis package PATN (Belbin, 1995) was used to ordinate the year 2000 crab hepatopancreas samples using tissue metal concentrations as the attributes to ordinate the samples. Prior to ordination, metal concentrations were standardised (*viz.* within each metal, the following equation was applied to each values: xnew = (x-xmin)/xrange) to produce a scale of 0 – 1 and thereby give each metal an equal weighting in the analysis. This was necessary to set all metals on the same scale and so avoid metals on a high scale having an over-bearing influence on the analysis.

Samples were ordinated using **S**emi-**S**trong **H**ybrid **M**ulti**d**imensional **S**caling (SSH MDS) to produce an n-dimensional scatter plot of samples. Similarity between samples was determined using the Bray-Curtis association measure. Samples were labelled according to the five groupings and these groupings were superimposed on the ordination plot to assess the distinctiveness of the groupings in ordination space. To test the significance of any separation of these groups of samples in ordination space, the **An**alysis **o**f **Sim**ilarity (ANOSIM) option in PATN (Belbin 1995) was invoked.

Finally, the Principal Axis Correlation (PCC) option in PATN was used to determine the significance and direction of gradients of metals through the ordination. Monte Carlo randomisations (n=100) of the data were performed to test the significance of these gradients (i.e. a gradient was taken as significant if the actual correlation coefficient of the gradient was greater than 95 of the 100 correlation coefficients produced from the randomisations of the data).

7.1.3.7 Hepatopancreas 2000 comparison; Fitzroy, Gladstone and Ayr

As a similar prevalence of shell disease existed in Fitzroy crabs a summary was made of comparisons of metal concentrations in female crab hepatopancreas samples from Fitzroy, Gladstone and Control (FZ, GS & CT) from 2000. Sites (Gladstone GS, Fitzroy FZ and Ayr (control) CT) and condition (Diseased DS and Non-diseased ND) were kept separate to give 4 levels in a one-way ANOVA design. An *a posteriori* Tukeys HSD multiple range test was used to locate differences between levels where a significant main effect was recorded.

7.1.3.8 Muscle analyses 1999

As there were several measurements (metals) for the same individual, a MANOVA (multivariate analysis of variance) was considered the appropriate analysis for

difference between diseased/ non-diseased and Ayr/Gladstone. All metals that were all or mostly < 0.01 were excluded from the analyses. If an overall result was significant then each metal was then treated separately in a one-way ANOVA design.

7.1.3.9 Muscle analyses 2000

A summary was made of comparisons of metal concentrations in crab muscle samples, from 2000 by sites (CT/GS), sexes (M/F) and condition (DS/ND). Prior to analysis, any metals with all values less than detection were deleted. This left 11 metals with at least one value above detection. For any remaining values reported as less than detection, half the detection level was taken as the value to be used in analysis (i.e. for a detection level of <0.1, a value of 0.05 was used). Sexes, sites and condition were kept separate to give 5 levels in a one-way ANOVA design. An *a posteriori* Tukeys HSD multiple range test was used to locate differences between levels where a significant main effect was recorded.

7.1.3.10 Muscle 1999 and 2000 comparison

The main aim of this analysis was to look for any increases or decreases in concentrations of Cu, Zn and Al, the main metals of interest, between 1999 and 2000. Therefore, and because of the unbalanced design and inconsistent differences between sexes, sites and condition reported earlier, analyses were targeted at comparing changes in concentrations of Cu, Zn and Al between years for each pair of comparable samples (i.e. (Ayr) CT-Males in 1999 versus CT-Males in 2000 and (Gladstone) GS-DS-Females 1999 versus GS-DS-Females 2000). One-way ANOVA was used to test for significant differences, and an *a posteriori* Tukeys HSD multiple range tests was used to locate differences between concentrations where a significant main effect was recorded.

7.2 RESULTS

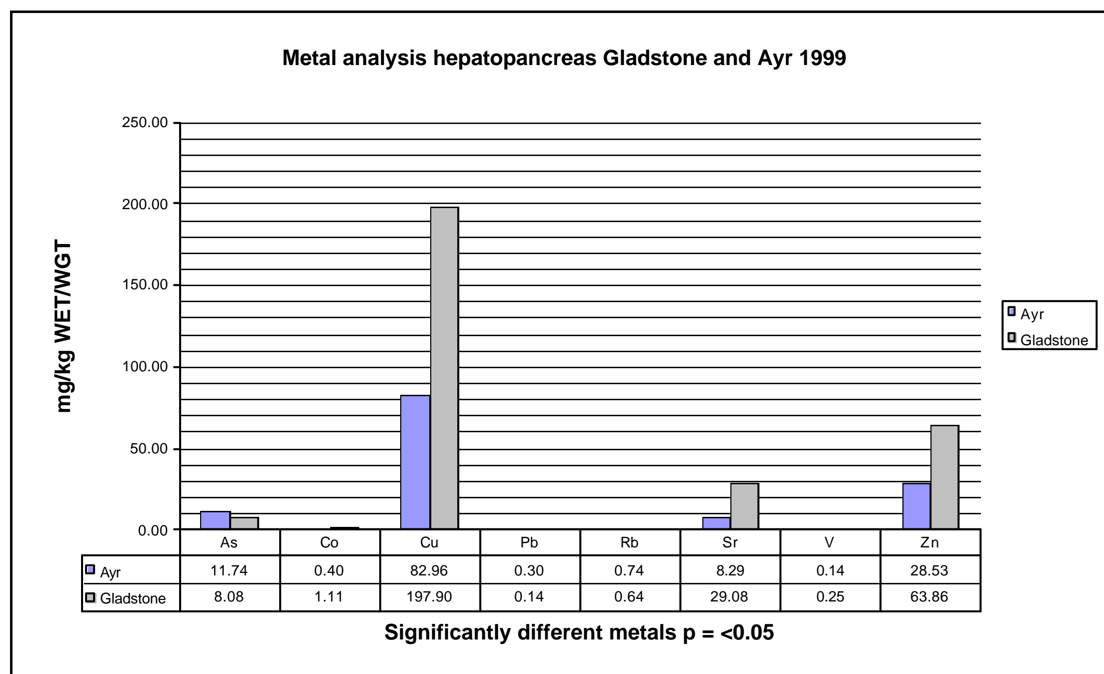
7.2.1 Metal analyses

A modified copy of the periodic table containing abbreviations and full names of all metals listed below is contained in Appendix 4. All results are on a wet weight basis (mg/kg).

7.2.1.1 Hepatopancreas analyses 1999

As there was no significant difference within the Gladstone sample (between diseased and non diseased crabs), these results were pooled and compared with the Ayr samples ($df = 18,1$ $F = 8.584$, $p = 0.263$). As, Co, Cu, Pb, Rb, Sr, V and Zn were all significantly different between Ayr and Gladstone samples. As and Rb were higher in Ayr crabs and the remainder were higher in Gladstone. Results are presented in Figure 7.2.

Figure 7.2. Metal analyses of mud crab hepatopancreas from Gladstone and Ayr 1999. Results are in mg/kg-wet wgt. Values are mean concentrations of each metal from each site.



	As	Co	Cu	Pb	Rb	Sr	V	Zn
Ayr	11.74	0.40	82.96	0.30	0.74	8.29	0.14	28.53
Gladstone	8.08	1.11	197.90	0.14	0.64	29.08	0.25	63.86

7.2.1.2 Hepatopancreas analyses 2000

A summary was made of comparisons of metal concentrations in 2000 crab hepatopancreas samples by sites (CT/GS), sexes (M/F) and condition (DS/ND). Results are presented in Table 7.1. Means and standard errors are tabulated to allow between-level comparisons (Appendix 5).

Of the 29 ANOVAs performed, 15 were significant, and 13 of those had a significant Tukey's result @ $p < 0.05$ and 9 @$p < 0.01$ (shown); several ANOVAs were close to being non-significant (Sn, $p = 0.033$ and Ba, 0.037) and Tukey's failed to detect any difference. For three metals (As, La & Nd), concentrations in hepatopancreas were significantly greater at one site than all other sites; for As, CT-F-ND was higher than all other sites, for La and Nd, GS-F-DS were higher than all other sites. In all other instances where significant main effects were recorded, there was overlap in concentrations between the five sites (i.e. concentrations at one site may be higher than at one other site but not different from the three remaining sites (i.e. Fe).

7.2.1.3 Hepatopancreas 1999 and 2000 comparison

The results are shown in Table 7.2 and the mean concentrations in Appendix 6. Only plots of metals showing no or negligible overlapping of confidence intervals are presented in Figure 7.3.

The only metal, for which concentrations at Ayr in both 1999 and 2000 were higher than at Gladstone in both years, was As. Metals for which concentrations at Gladstone in both 1999 and 2000 were higher than at Ayr in both years were Cu and Zn. For most metals there was only one site/year that was different from only one of the other site/years. In a few instances, one site/year was different from all the other site/years (e.g. Ce, La, Nd).

Table 7.1. One-way ANOVA on yr 2000 crab hepatopancreas metal concentrations from Control (CT) and Gladstone (GS) for Male (M) and Female (F) crabs, Non-diseased (ND) and Diseased (DS) (degrees of freedom = 4,74). Data were log (x+1) transformed prior to analysis. Tukey's multiple range tests were applied to locate differences between levels for significant main effect. Levels joined by a common line are not significantly different at p = 0.05. Refer to Appendix 6 for means and standard errors for each metal at each level (metals which were not significant are not shown).

Metal	F	p	Tukeys HSD Multiple Range Test				
As	16.72	0.0001	CT-F-ND	CT-M-ND	GS-M-ND	GS-F-ND	GS-F-DS
Ce	6.94	0.0001	GS-F-DS	GS-M-ND	CT-M-ND	GS-F-ND	CT-F-ND
Cr	7.71	0.0001	GS-F-DS	GS-F-ND	GS-M-ND	CT-F-ND	CT-M-ND
Cu	15.99	0.0001	GS-M-ND	GS-F-ND	GS-F-DS	CT-M-ND	CT-F-ND
Fe	3.63	0.0095	CT-F-ND	CT-M-ND	GS-F-ND	GS-M-ND	GS-F-DS
Hg	5.42	0.0001	CT-F-ND	GS-F-DS	CT-M-ND	GS-M-ND	GS-F-ND
La	6.88	0.0001	GS-F-DS	GS-M-ND	CT-M-ND	GS-F-ND	CT-F-ND
Nd	5.75	0.0001	GS-F-DS	GS-M-ND	GS-F-ND	CT-M-ND	CT-F-ND
Zn	6.76	0.0001	GS-F-ND	GS-M-ND	CT-M-ND	GS-F-DS	CT-F-ND

7.2.1.4 Hepatopancreas within group variation 1999 and 2000

Metal concentrations in crabs in 2000 were more variable than in crabs in 1999 (Table 7.3). Also, metal concentrations in diseased Gladstone (DS) crabs were more variable than in Ayr control (CT) crabs, but neither was different from Gladstone non-disease (ND) crabs. There was a significant interaction term that indicates this was not consistent across years. This significant interaction was because DS crabs had the highest mean coefficient of variation (CV) in 2000 but the lowest mean CV in 1999 (Figure 7.4).

Table 7.2 Metals in mud crab hepatopancreas in 1999 and 2000 for which a significant effect was recorded, giving chi-square statistic and level of significance.

Metal	Chi-sq	Significance
As	36.2	<0.0001
Cd	24.5	<0.0001
Ce	13.4	0.0039
Co	27.0	<0.0001
Cr	38.4	<0.0001
Cu	42.1	<0.0001
Fe	12.1	0.0071
Hg	22.06	<0.0001
La	15.3	0.0016
Mn	17.3	0.0006
Nd	14.7	0.0021
Pb	47.0	<0.0001
Rb	100.6	<0.0001
Sn	11.3	0.0104
Sr	25.7	<0.0001
U	15.8	0.0012
V	30.6	<0.0001
Zn	29.4	<0.0001

Table 7.3. Two-way ANOVA testing between year and group differences in coefficient of variation (CV) in metal concentrations of mud crab hepatopancreas from Gladstone diseased (DS) and non-diseased (ND) and Ayr controls (CT), with Tukeys multiple range test of between-group differences in mean CV, using metals as replicates. A common line joins groups not significantly different from each other. Groups are arranged in descending order. Mean CV for each level are in parenthesis. Mean CV is plotted in Figure 7.3.

Level	df	F	P	Tukeys Range Test		
Year	2	3.9	0.023	2000 (71.8)	>	1999 (46.1)
Group	1	11.8	0.0008	DS (73.4)	ND (54.7)	CT (48.9)
Year * Group	2	4.9	0.0089			

Figure 7.3. Mean (+/- 95% confidence intervals) concentration (mg/kg wet wgt) of metals in crab hepatopancreas for Ayr99 and Ayr00 (Ayr 1999 and 2000) and Glad99 and Glad00 (Gladstone 1999 and 2000).

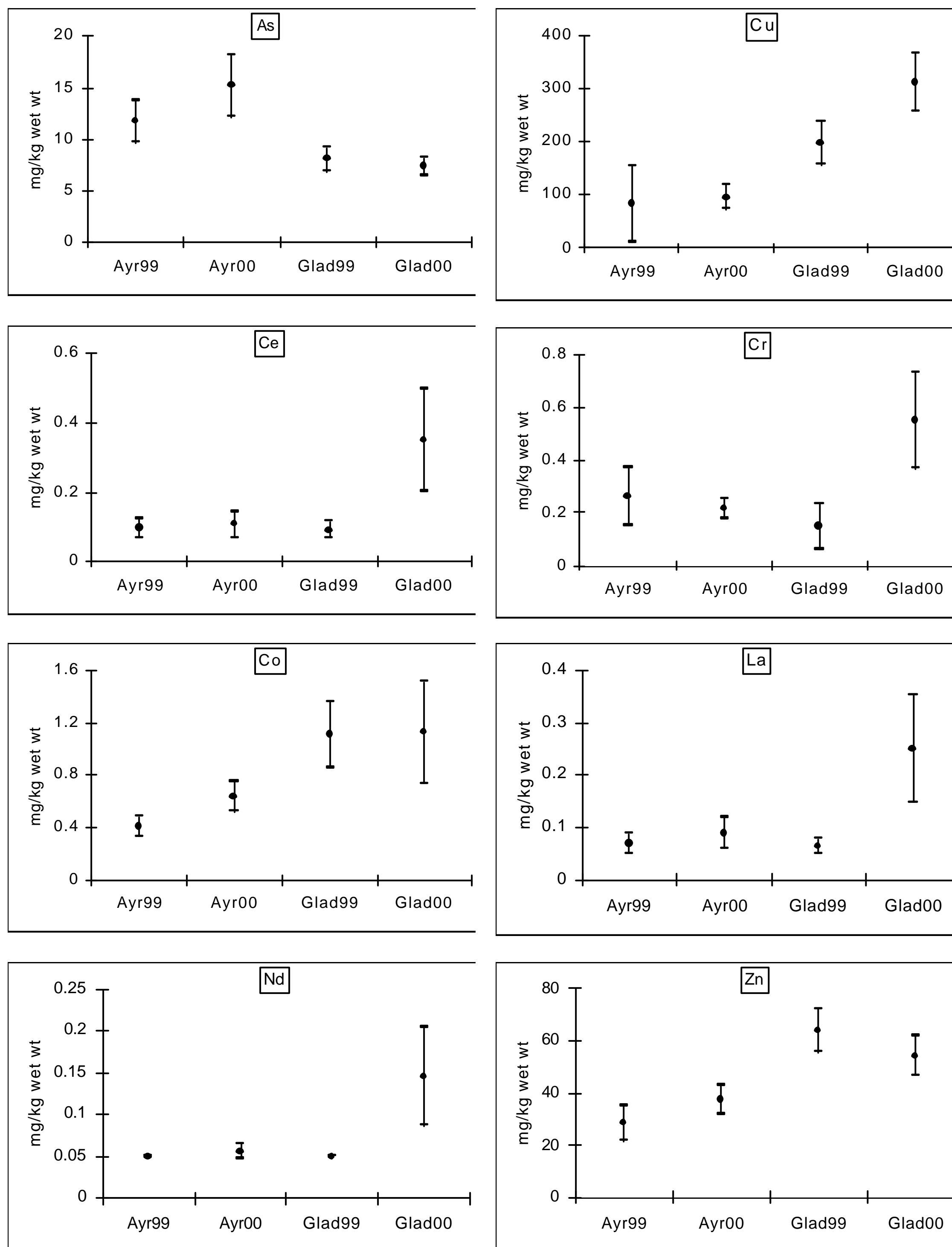
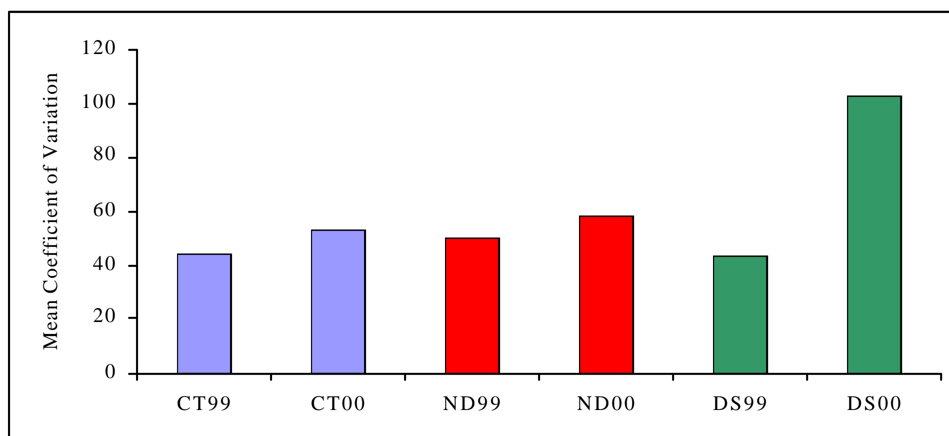


Figure 7.4. Mean Coefficient of Variation (CV) for each year/group for mud crab hepatopancreas control (CT) disease (DS) and non-disease (ND) in 1999 and 2000.



7.2.1.5 Hepatopancreas 2000 metal burdens

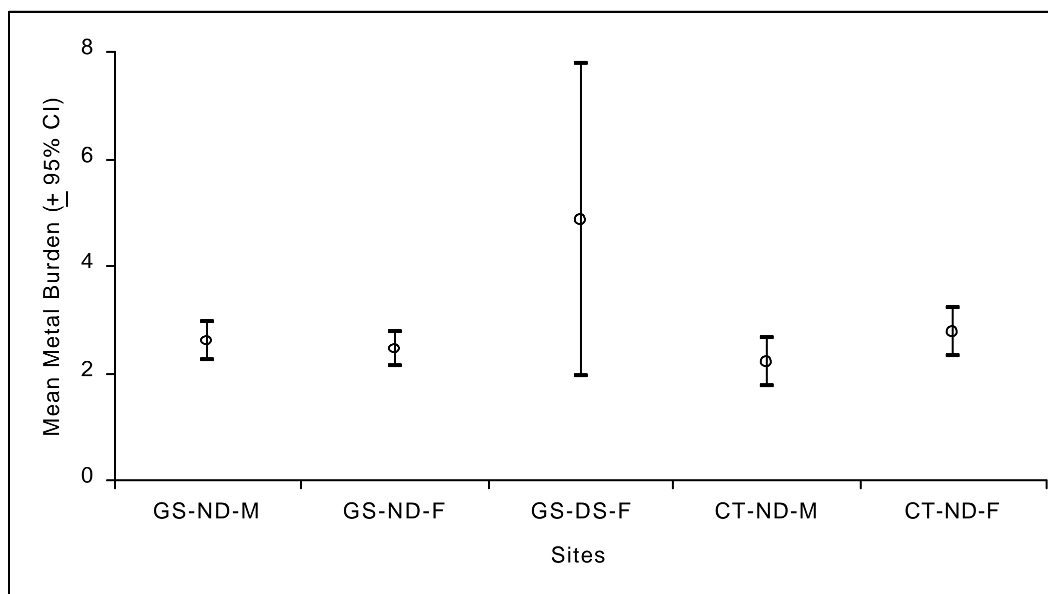
The GS-F-DS group of samples had a high within sample variance compared to the other four groups. A log10 (x+1) transformation gave the best approximation to homogeneity of variances, but Bartletts test remained significant due to the influence of the high variance of the GS-F-DS sample. Therefore, results of the ANOVA (Table 7.4) should be treated with caution. Mean metal burden ($\pm$ 95% CI) for each group are illustrated in figure 7.5.

ANOVA failed to detect a significant between-group difference in total metal burden. As can be seen from Figure 7.5 the mean metal burden in the Gladstone Diseased Female crabs (GS-DS-F) was much higher than all other groups but with high within-group variability, which prevented any difference from being significant. Within the remaining four groups there was no obvious between-group differences in total metal burden, and within-group variability was relatively low.

Table 7.4. One-way ANOVA on metal burden in yr 2000 crab hepatopancreas from Gladstone and Ayr (GS/CT) for Male/Female (M/F) diseased/non-diseased (DS/ND) crabs (degrees of freedom = 4,74). Tukey's multiple range tests were applied to locate differences between sites for a significant main effect. Levels joined by a common line are not significantly different at p = 0.05. Refer to Figure 7.5 for means and 95 % Confidence Intervals for metal burden in each group.

Metal	F	p	Tukeys HSD Range Test				
Log. Burden	1.39	ns	GS-DS-F	CT-ND-F	GS-ND-M	GS-ND-F	CT-ND-M

Figure 7.5. Mean metal burden (± 95 % CI) for yr 2000 crab hepatopancreas samples from Gladstone (GS) and Ayr (CT) for Male/Female (M/F), Diseased/Non-diseased crabs (DS/ND).



7.2.1.6 Hepatopancreas 2000 multivariate analyses

Ordination of the 75 samples using the 28 metals produced an optimal solution of a 3-dimensional ordination with a stress (*viz.* goodness of fit) value of 0.1752. This stress value is relatively high (NB a stress value < 0.15 is desirable and > than 0.2 is considered unacceptable), but was considered as acceptable and indicates a degree of variability (noise) in the data. Stress could be further reduced by introducing a 4th dimension to the ordination – but this would complicate interpretation, and therefore interpretation is based upon a 3-dimensional output.

Superimposing the five sample groupings on the ordination indicated a high degree of overlap within all Gladstone samples, and overlap within all Ayr samples, but there was relatively good separation of Gladstone samples from Ayr (Figure 7.6). ANOSIM detected a highly significant ($p<0.0001$) separation of groups, indicating that one or more of the five groups was well separated from the other groups in ordination space.

Principal Axis Correlation (PCC) determined that all 28 metals had significant gradients through the ordination space, although gradients for some metals were more significant as determined by the respective correlation coefficients (r-values; see Table 7.5). All gradients mostly were in the same direction indicating elevated concentrations in the Gladstone samples, particularly several of the GS-DS-F samples. Several metals had gradients in the direction of the Ayr samples (Cd, Hg, Fe, Sr, As) indicating elevated concentrations of these metals in Ayr compared to Gladstone samples. Cu and Zn showed a strong gradient directly away from the Ayr samples, indicating strongly elevated concentrations in Gladstone samples and these differed from the other metals, as their gradients seemed to be influenced by several GS-DS-F samples.

Figure 7.6. Vec 3 by Vec 2, and Vec 1 by Vec 3 MDS ordination plots of the 75 yr 2000 crab hepatopancreas samples based on standardised concentrations of the 28 tissue metals within each sample. Samples are grouped according to site (Gladstone = shaded symbols, Ayr = open symbols), condition (Diseased = Red, Non-diseased = Blue) and sex (Male = triangles , Female = square). Balloons approximately enclose Ayr and Gladstone samples in the Vec 3 by Vec 2 plot. Gradients of metals through the ordination are presented for the Vec 1 by Vec 3 plot. Refer to Table 7.6 for metals in the general "Metal Gradient A".

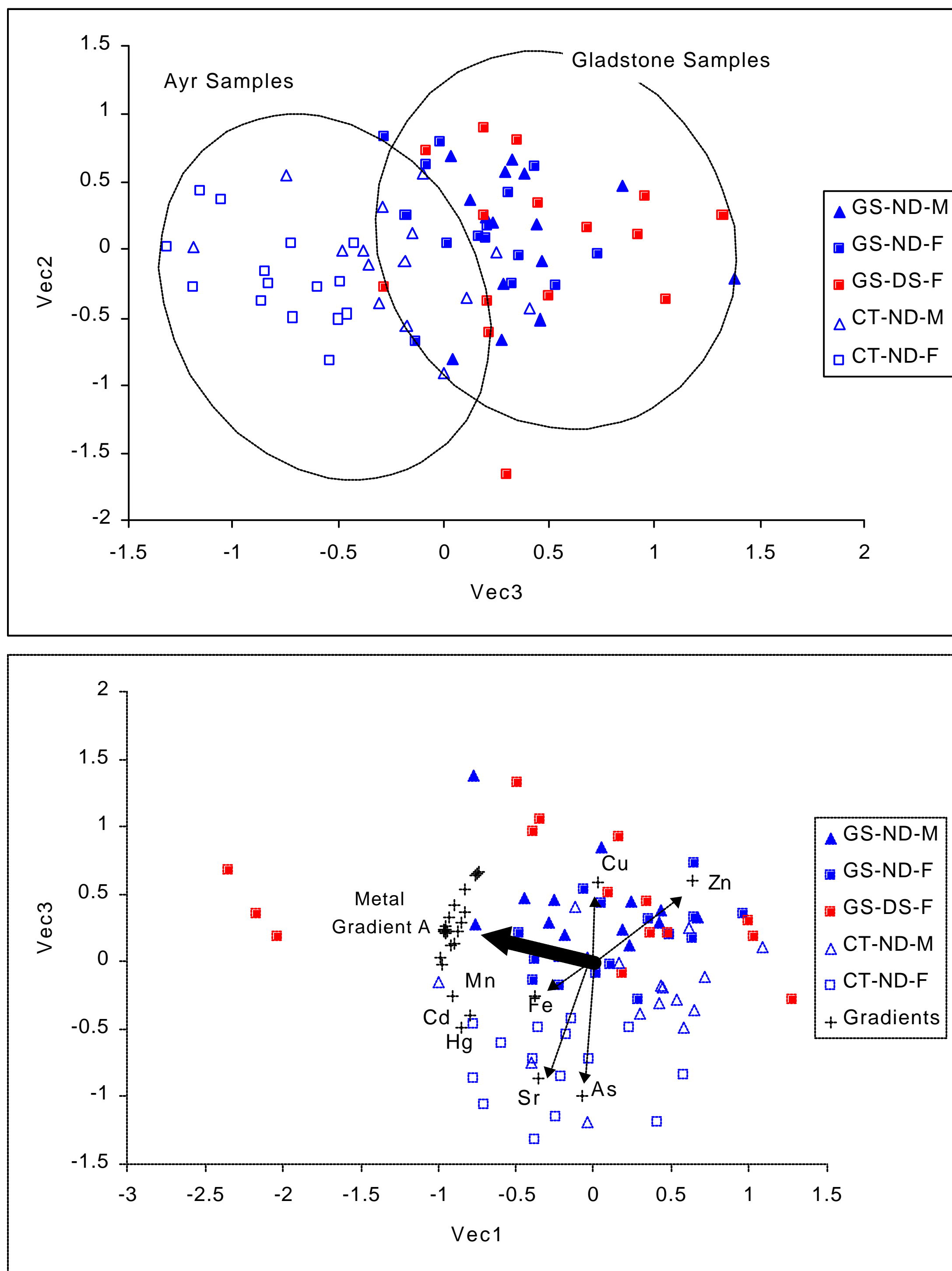


Table 7.5. Correlation coefficients of the gradients of each of the 28 metals through the ordination of the 75 hepatopancreas samples. The greater the r-value indicates the stronger the gradient.

Metal	r-value	Metal	r-value
La	0.814	**Sr**	0.656
Hg	0.814	**Cr**	0.654
Ce	0.813	**Pr**	0.651
Nd	0.787	**Sn**	0.634
Cd	0.777	**Ba**	0.621
Co	0.741	**Gd**	0.599
V	0.741	**Al**	0.598
Mn	0.736	**Sb**	0.577
Cu	0.717	**Zn**	0.573
Ni	0.716	**U**	0.557
Ag	0.712	**Sm**	0.556
Mo	0.693	**Fe**	0.546
As	0.684	**Ga**	0.431
Pb	0.671	**Te**	0.365

Table 7.6. Metals in the general "Metal Gradient A", as illustrated in the Vec 1 by Vec 3 ordination plot in Figure 7.6.

Ag, Al, Ba, Ce, Co, Cr, Ga, Gd, La, Mo, Nd, Ni, Pb, Pr, Sb, Sm, Sn, Te, U, V

7.2.1.7 Hepatopancreas 2000 comparison; Fitzroy, Gladstone and Ayr

Results are recorded in Table 7.7. Means and standard errors are tabulated to allow between-level comparisons (Appendix 7).

Of the 29 ANOVAs performed, 14 were significant, 13 of which had a significant Tukey's result @ $p > 0.05$. For five metals (As, Ce, La, Nd and Ni), concentrations in hepatopancreas were significantly greater at one or more sites than all other sites. There was no consistent trend of any site having the highest concentration. For one metal (Cu) one site was significantly less than all other sites (CT-ND).

7.2.1.8 Muscle analyses 1999

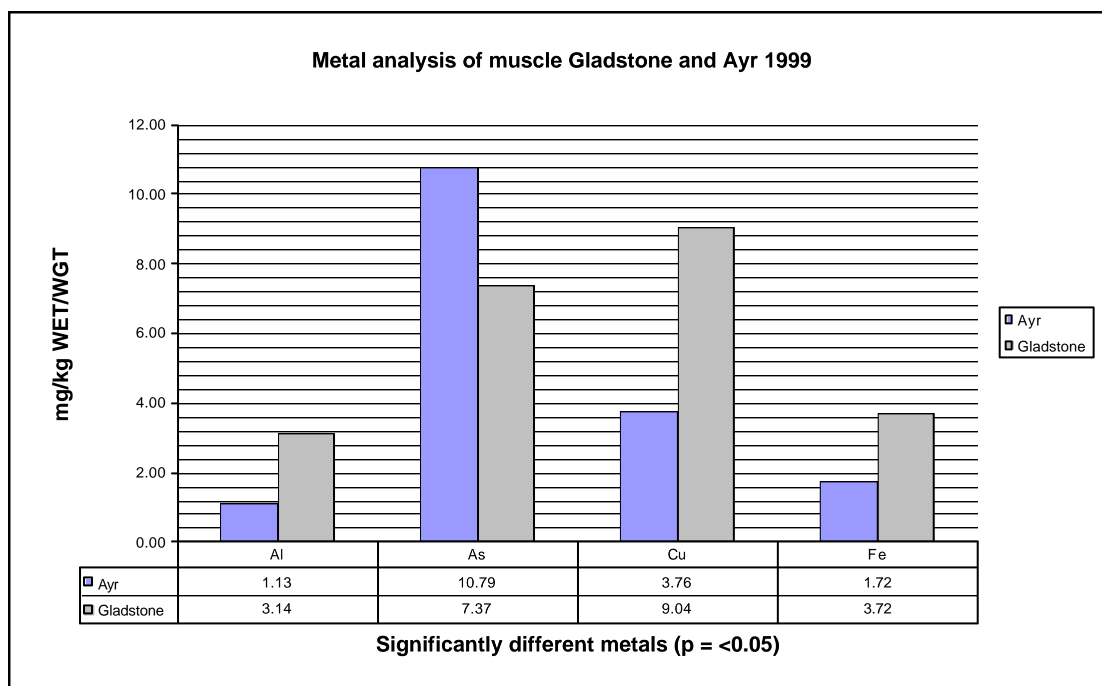
As there was no significant difference within the Gladstone sample (between diseased and non diseased crabs), these results were pooled and compared with the Ayr samples (df =9, F = 1.638, 10, p= 0.226). In muscle tissue the metals, which were significant among sites were Al, As, Cu and Fe. As in hepatopancreas, As was higher in Ayr than Gladstone whereas the rest were higher in Gladstone. Means of significant metals are illustrated in Figure 7.7.

Table 7.7. One-way ANOVA on yr 2000 female crab hepatopancreas metal concentrations from Control (CT), Fitzroy (FZ) and Gladstone (GS) for Non-diseased (ND) and Diseased (DS) crabs (degrees of freedom = 3,53). Data were log (x+1) transformed prior to analysis. Tukey's multiple range tests were applied to locate differences between levels for significant main effect. Levels joined by a common line are not significantly different at p = 0.05. Refer to Appendix 7 for means and standard errors for each metal at each level.

Metal	F	p	Tukeys HSD Multiple Range Test			
Ag	6.31	0.0001	FZ-ND	GS-DS	GS-ND	CT-ND
Al	2.05	ns				
As	21.69	0.0001	CT-ND	FZ-ND	GS-ND	GS-DS
Ba	4.36	0.0084	FZ-ND	GS-DS	GS-ND	CT-ND
Cd	1.45	ns				
Ce	8.60	0.0001	GS-DS	FZ-ND	GS-ND	CT-ND
Co	1.62	ns				
Cr	4.76	0.0054	GS-DS	GS-ND	CT-ND	FZ-ND
Cu	14.44	0.0001	GS-ND	FZ-ND	GS-DS	CT-ND
Fe	3.50	0.02	CT-ND	GS-ND	FZ-ND	GS-DS
Ga	0.86	ns				
Gd	1.83	ns				
Hg	3.91	0.0139	CT-ND	GS-DS	FZ-ND	GS-ND
La	8.61	0.0002	GS-DS	FZ-ND	GS-ND	CT-ND
Mn	2.33	ns				
Mo	0.57	ns				
Nd	6.96	0.0005	GS-DS	FZ-ND	GS-ND	CT-ND
Ni	4.39	0.0081	FZ-ND	CT-ND	GS-DS	GS-ND
Pb	2.46	ns				
Pr	3.35	0.0262	CT-ND	FZ-ND	GS-DS	GS-ND
Sb	1.80	ns				
Sm	1.75	ns				
Sn	2.34	ns				
Sr	3.37	0.0257	CT-ND	FZ-ND	GS-ND	GS-DS
Te	0.86	ns				

U	0.66	ns	
V	1.58	ns	
Zn	8.09	0.0002	GS-ND FZ-ND GS-DS CT-ND

Figure 7.7. Metal analyses of mud crab muscle from Gladstone and Ayr 1999. Results are in mg/kg-wet wgt.



	Al	As	Cu	Fe
Ayr	1.13	10.79	3.76	1.72
Gladstone	3.14	7.37	9.04	3.72

7.2.1.9 Muscle analyses 2000

Of the eleven tests performed, seven were significant. However, there was no consistent pattern of either high or low metal concentrations in any sample. Al was the only metal which was significantly higher in all groups of Gladstone crabs compared to control groups. Results are tabulated in Table 7.8. Refer to Appendix 8 for means and standard errors for each metal at each level.

Table 7.8. One way ANOVA on yr2000 crab muscle metal concentrations from Control (CT) and Gladstone (GS) for Male (M) and Female (F) crabs, Non-diseased (ND) and Diseased (DS) (degrees of freedom = 4,49). Data were log(x+1) transformed prior to analysis. Tukey's multiple range tests were

applied to locate differences between levels for significant main effect. Levels joined by a common line are not significantly different at p = 0.05.

Metal	F	p	Tukeys HSD Multiple Range Test				
Al	38.49	<0.0001	GS-M-ND	GS-F-ND	GS-F-DS	CT-M-ND	CT-F-ND
As	9.23	<0.0001	CT-F-ND	GS-F-ND	GS-F-DS	CT-M-ND	GS-M-ND
Ba	1.27	ns					
Cr	0.59	ns					
Cu	7.73	<0.0001	CT-F-ND	GS-F-DS	GS-M-ND	GS-F-ND	CT-M-ND
Fe	4.25	0.005	GS-M-ND	GS-F-ND	GS-F-DS	CT-M-ND	CT-F-ND
Hg	8.16	<0.0001	CT-F-ND	GS-F-ND	CT-M-ND	GS-F-DS	GS-M-ND
Mn	1.82	ns					
Sr	2.41	ns					
V	7.83	<0.0001	GS-M-ND	CT-M-ND	GS-F-DS	GS-F-ND	CT-F-ND
Zn	11.70	<0.0001	GS-F-ND	CT-F-ND	GS-F-DS	GS-M-ND	CT-M-ND

7.2.1.10 Muscle 1999 and 2000 comparison

Results of ANOVA are presented in Table 7.9 and means and standard errors are tabulated to allow between-level comparisons in Appendix 9. There were no between-year differences in the concentration of Cu in crabs from any group (site/sex/condition), although there was a trend for Cu in samples from 2000 to be higher than 1999. However, the relatively high within sample variance precluded these differences from being significant. Similarly, there was no between-year difference in the concentration of Zn in male crabs from control sites. However, for all crabs from Gladstone (male and female, diseased and non-diseased) there was a consistent pattern of higher tissue Zn concentrations in 2000 compared to 1999. For Al, there were no between-year differences in concentrations in male crabs from control sites, or female crabs (DS and ND) from Gladstone, however, ND Male crabs from Gladstone had higher concentrations of Al in 2000 compared to 1999.

Table 7.9. One-way ANOVAs on between-year (yr 1999 and yr 2000) differences in crab muscle Cu, Zn and Al concentrations, from Control (CT) and Gladstone (GS) for Male (M) and Female (F) crabs, Non-diseased (ND) and Diseased (DS). Data were log (x+1) transformed prior to analysis. Tukey's multiple range tests were applied to locate differences between years for significant main effect.

Level	Metal	df	F	p	Tukeys Multiple Range Test		
CT-Males (ND)	Cu	1,19	2.35	ns	1999	=	2000
	Zn	1,19	1.37	ns	1999	=	2000
	Al	1,19	0.13	ns	1999	=	2000
GS-Males (ND)	Cu	1,13	0.19	ns	1999	=	2000
	Zn	1,13	8.51	0.012	2000	>	1999
	Al	1,13	19.89	<0.0001	2000	>	1999
GS-Females (ND)	Cu	1,15	3.41	ns	1999	=	2000
	Zn	1,15	49.99	<0.0001	2000	>	1999
	Al	1,15	3.38	ns	1999	=	2000
GS-Females (DS)	Cu	1,17	2.53	ns	1999	=	2000
	Zn	1,17	20.01	<0.0001	2000	>	1999
	Al	1,17	0.10	ns	1999	=	2000

7.3 DISCUSSION

7.3.1 Metal analyses

The results of each tissue metal analyses will be discussed individually with an overview presented in a conclusion at the end of this section.

7.3.1.1 Hepatopancreas analyses 1999

The results of metal analyses of hepatopancreas tissue highlights copper and zinc in particular as being elevated in Gladstone compared to Ayr. As each group of crabs (diseased, non-diseased and control) was of mixed gender, analyses were repeated in 2000 in order to determine if there were gender differences in metal concentrations.

7.3.1.2 Hepatopancreas analyses 2000

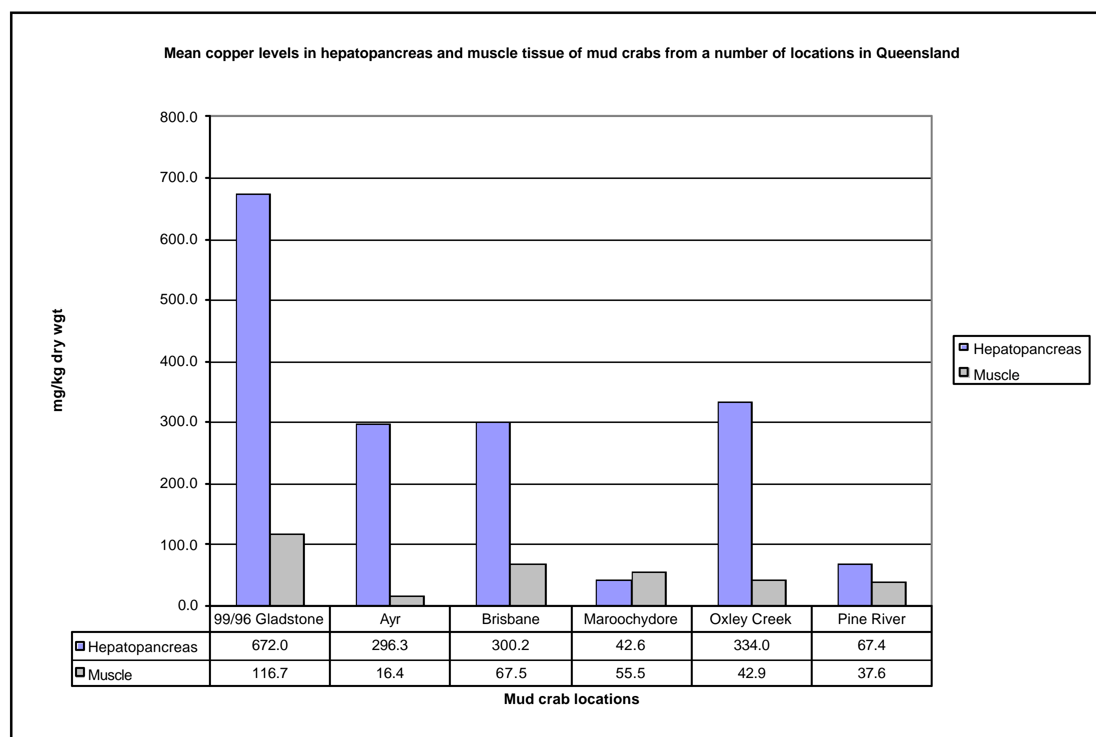
By separating the results by gender as well as site and condition, an attempt was made to determine if there was a gender difference in metal accumulation, as gender differences had been noted previously in the prevalence of shell disease as well as the areas of the shell affected by shell disease. In many instances, there was a trend for metal concentrations in hepatopancreas from one or more of the three Gladstone sites (GS-M-ND, GS-F-ND or GS-F-DS) to be higher than Control sites (i.e. Ce, Cr, Cu, La, Nd, Zn) and in a few instances, the Gladstone sites tended to be lower than the Control sites (i.e. As, Fe, Hg,), however, these differences were never consistent, and all Gladstone or all Control sites were never all higher/lower than the other sites. The establishment of a significant result, however, was confounded by the high variation

in metal concentrations in the diseased female group only. The only metals therefore which were significantly higher in Gladstone diseased crabs compared to all other groups was the rare earth metals; La and Nd. Interestingly, Al which was significant in muscle samples, did not feature in hepatopancreas samples.

7.3.1.3 Hepatopancreas 1999 and 2000 comparison

Concentrations of Cu and Zn were again shown to be higher in Gladstone crabs compared to Ayr crabs in 2000 as they were in 1999. Mean concentrations of Cu were more than three times higher in Gladstone crabs in 2000 compared to Ayr crabs (in comparison to just over two fold for 1999). Mean concentrations of Zn were just under twice the value of the Ayr crabs for 2000. The mean hepatopancreas and muscle copper concentrations for mud crabs from a number of locations in Queensland (including Gladstone in 1996) have been determined by Mortimer (2000) and are also shown to be lower than average values established for Gladstone crabs in either 1999 or 2000 (figure 7.8). A statistical comparison between combined Gladstone 96/99 hepatopancreas concentrations (there was no significant difference between the copper concentrations in 1996 (Mortimer 2000) compared to 1999 $df = 1,28$, $F = 0.290$, $p = 0.594$) and three other locations (Brisbane, Maroochydore and Ayr) determined that the only metal that was significantly elevated in Gladstone mud crab hepatopancreas compared to the other sites, was copper ($df = 4$, $F = 25.492$, $p = < 0.0001$). Concentrations in Gladstone were more than twice as high as the urbanized Brisbane site.

Figure 7.8 Copper concentrations in hepatopancreas and muscle tissue from a number of locations in Queensland. Results are in mg/kg dry wgt.



	99/96 Gladstone	Ayr	Brisbane	Maroochydore	Oxley Creek	Pine River
Hepatopancreas	672.0	296.3	300.2	42.6	334.0	67.4
Muscle	116.7	16.4	67.5	55.5	42.9	37.6

Gladstone n=30, Ayr n=10, Brisbane n=16, Maroochydore n=35, Oxley Creek n=8, Pine River n=8. Adapted from Mortimer (2000).

The As concentrations were higher in Ayr crabs than they were in Gladstone crabs, in 2000. A similar result was found in 1999. Apart from these three metals, however,

there is no consistent trend of metal concentrations in crabs from Gladstone in both years being higher or lower than concentrations in crabs from Ayr, or of a consistent pattern of 1999 levels being higher or lower than 2000. The rare earth metals La, Ce and Nd, which featured in Gladstone 2000 samples, were not significant in 1999 and perhaps should be treated with caution.

7.3.1.4 Hepatopancreas metal burden and within group variation

Tukey's range test was unable to locate a significant difference among sites due to the high within group variability in metal concentrations that exists in the Gladstone diseased crabs, although the mean metal burden for Gladstone diseased female crabs was obviously much higher than any other group including their Gladstone non diseased counterparts. This high within group variability was also highlighted in the muscle tissue of Gladstone crabs compared to Controls and confounded efforts to establish significant differences between groups in a number of metals in both tissue types. This was particularly apparent with the rare earth metals (Ce, La, Nd, and Pr) in a comparison between diseased and non-diseased female hepatopancreas 2000 samples from Gladstone. In all cases concentrations and variability were higher in DS than ND. This high with-in group variability for GS-F-DS was due to very high concentrations in several crabs and much lower concentrations in the remaining specimens for this group.

7.3.1.5 Hepatopancreas 2000 multivariate analyses

The results of the ordination plots were similar to those of the metal burden analyses and highlighted elevated metal concentrations in Gladstone crabs, particularly the Gladstone diseased female crabs in comparison to the Ayr crabs. In particular Cu and Zinc showed a strong gradient towards the Gladstone samples. The gradient of many metals was influenced again by the high variability that was seen in the Gladstone diseased female samples.

7.3.1.6 Hepatopancreas 2000 comparison; Fitzroy, Gladstone and Ayr

It was established that a similar prevalence of shell disease existed in crabs from the Fitzroy River area as crabs from Port Curtis sampled over the same period of time (Chapter 3). As it is possible that the Fitzroy/Port Curtis mud crabs belong to one large population and that mixing of crabs from the two areas occurs via The Narrows which connects the two water bodies, metal analyses was undertaken on the Fitzroy crabs to determine if the concentrations were comparable to Port Curtis.

Copper was again the only metal which was significantly higher in Gladstone and Fitzroy crabs compared to Ayr. Hepatopancreas concentrations of Ni in female crabs was greater in Fitzroy than all other sites, and in two other instances (Ag and Ba), concentrations at Fitzroy were greater than at least one other site including Ayr. Again the rare earth metals (Ce, La and Nd) were higher in Gladstone diseased crabs compared to all other sites. Interestingly, As concentrations, which were significantly higher in Ayr in 1999 and 2000 compared to Gladstone, were not significantly different in Fitzroy crabs compared to Ayr. For approximately half of those tests that were significant, samples from the Control site had the lowest mean metal concentrations. It appears that metal burdens in Fitzroy crabs are also elevated compared to the Control site. The association of metal concentrations and shell disease will be discussed in Chapter 10.

7.3.1.7 Muscle analyses 1999

Again copper among other metals (Al, Fe, Ba and Rb) was highlighted as being elevated in the Gladstone samples compared to Ayr. Metal analyses were also repeated in 2000 to establish gender differences.

7.3.1.8 Muscle analyses 2000

Crabs from control sites were not consistently higher or lower than those from Gladstone. Similarly, there was no consistent pattern of male crabs being higher or lower than females or of diseased crabs being higher or lower than Non-diseased crabs. For Al, however, control crabs were lower than those from Gladstone. Al was also significantly lower in control crabs compared to Gladstone crabs in 1999, along with Cu, Fe, Ba and Rb. However, these latter metals did not feature in the 2000 data.

7.3.1.9 Muscle 1999 and 2000 comparison

As Gladstone is an industrial port, comparisons were made to determine if metal accumulations in mud crabs changed over successive years. A large within group variation, however, again prevented establishment of a significant difference among some groups. Although there was a trend for increasing concentrations of Cu, Zn and Al in some groups of crabs, these results should be interpreted with caution. Apart from anthropogenic inputs, a range of environmental factors including climatic factors, could also affect perturbations in metal accumulations in biota at industrially impacted sites. These accumulations may take years to become apparent through repeated sampling.

7.4 SUMMARY

Our findings indicate generally elevated metal concentrations in Gladstone (and Fitzroy) mud crabs compared to those from a non-impacted control site. When separating the results of the hepatopancreas 2000 metal analyses by gender as well as site and condition, an attempt was made to determine if there was a gender difference in metal accumulation, as gender differences had been noted previously in the prevalence of shell disease (Andersen et al. 2000). Significant outcomes, however, were confounded by the high within group variability in metal concentrations in the diseased group of mud crabs. Nevertheless, within gender comparisons emphasised copper as being the only metal elevated in Gladstone crabs compared to their same sex counterparts from the control site. Concentrations of both Cu and Zn, however, were shown to be significantly higher in a combined Gladstone group of crabs compared to Ayr crabs in 2000 as they were in 1999. Mean concentrations of hepatopancreas Cu were more than three times higher in Gladstone crabs in 2000 compared to Ayr crabs and were also significantly elevated in comparison to mud crabs from other pristine and impacted reference sites in Queensland (Mortimer 2000).

Feeley (1983) recorded a correlation between elevated copper (range 50-730mg/kg dry wgt) in the hepatopancreas and both the severity of shell lesions and the carapace size of the deep-sea red crab (*Chaceon quinquedens*). A significant difference between copper concentrations in the diseased and non-diseased group from Gladstone, however, could not be established in our study. This is possibly due to the assigning of a crab to either the diseased or non-diseased group, being qualitative (i.e. the overt presence of a lesion) rather than quantitative. Histology has demonstrated

that rust spot lesions are formed in the post moult phase only and are most likely caused by a defect in the manufacturing process of the endocuticular layer, with the proposed aetiology being an inhibition of calcium uptake (Andersen et al. 2000). A crab must moult therefore, to form a rust spot lesion. Two crabs (one designated as diseased and the other as non-diseased) could have similar hepatopancreas copper accumulations. The second crab, however, which is potentially "diseased" may not develop a lesion until its next moult. The possibility of copper being implicated in the cause of shell disease is discussed further in Chapter 8.

Multivariate analyses provided an alternate method for the examination of results and highlighted the generally elevated metal burdens in Gladstone crabs, in particular the Gladstone diseased crabs, in comparison to the Ayr crabs. In particular, copper and zinc showed a strong gradient towards the Gladstone samples. Both Weinstein et al. (1992) and Feeley (1983) also recorded elevated metal burdens in the diseased crabs they studied from contaminated areas compared to non-diseased crabs from reference sites. The gradient of many metals was influenced by the high variability that was seen in the Gladstone diseased samples. The variability also affected the outcomes of the metal burden analyses and although the mean metal burden for Gladstone diseased female crabs was obviously much higher than any other group including their Gladstone non-diseased counterparts, we were unable to locate a significant difference in metal burdens among sites. The reason for the high within group variability in diseased female crabs compared to all other groups is unknown, but suggests that some crabs in this group of crabs are perhaps unable to regulate metal concentrations. Feeley (1983) also commented that trace metal values varied considerably among the individual diseased crabs he studied, making intersite contaminate comparisons difficult. Decapod crustaceans possess a number of mechanisms to regulate the detoxification, storage and/or excretion of trace metals to maintain normal physiological functioning (Rainbow 1988). Therefore, although the biological relevance of higher variability in metal concentrations has not been determined, it could indicate some level of stress in the diseased group of crabs.

Although Al was found to be elevated in muscle tissue of Gladstone crabs in 1999 and 2000, it did not feature in hepatopancreas samples from either year. Cu, the main metal of interest in hepatopancreas for both years was elevated in muscle in 1999 only. Bjerregard and Vislie (1986) recorded that crabs (*Carcinus maenas*) exposed to Cu for 29 days accumulated Cu in hepatopancreas among other tissues, but not significantly in muscles and haemolymph. Other authors have reported not only species differences in metal accumulations (Harris and Santos 2000) but also within species differences in tissue affinities for metals (Arumugam and Ravindranath 1983, Mortimer 2000).

Although the mean concentrations of some metals in muscle tissue for both years were elevated, concentrations were not above new National Food Authority (ANZFSC 2000) standards, which were gazetted in December 2000. Although a maximum permitted concentration (MPC) for copper was removed from the new standards, the MPC for copper prior to this date was set at 10mg/kg-wet wgt (ANZFA 1999a). Mean level of copper in muscle tissue in 1999 was slightly lower than this level (9.9mg/kg), however, 35% of individuals were above this level. The mean level of copper in male mud crab muscle tissue for 2000 was 13.150mg/kg. Never the less, the Australia New Zealand Food Standards-Proposal 157 (1999b), which was part of

the process of the review of the Food Standards Code recommended that the MPC's for copper in foods be deleted due to "there being no cause for concern in terms of public health and safety from current dietary exposures to copper". The recommendation was based on the following findings outlined in the risk assessment:

? The toxicological endpoint (gastrointestinal disturbances), on which the PTDI for copper is based, is not life threatening.

? Humans have a capacity to maintain homeostasis in terms of copper concentrations by a combination of decreased absorption and increased excretion, thereby compensating for excessive intakes of copper.

? Toxicity is generally reversible once excessive doses of copper are withdrawn from the diet.

There have been very few occasional acute cases of copper poisoning in normal healthy populations (ANZFA 1999b). Consequently there is no risk of copper poisoning from consumption of Port Curtis mud crabs.

To bridge the information gap that may have resulted from removing some MPC's, Generally Expected Levels (GEL's) for some metals (Sb, As, Cu, Hg, Se and Zn) have been proposed by ANZFA as a draft guideline to the food standards code. The GEL's provide information on the 'normally' expected range of metal contaminants in food, but unlike the MPC's are not legally enforceable. Some metal concentrations in mud crab tissue for some metals are above these proposed GEL's and a continual review process of this guideline is therefore recommended.

Conclusion

Metal concentrations in particular copper and zinc are generally elevated in Gladstone (and Fitzroy River) mud crabs, compared to a non-impacted control site (Ayr). Levels of copper in mud crab hepatopancreas were also elevated compared to other locations sampled in Queensland. Total metal burdens in Gladstone mud crab hepatopancreas were also elevated compared to those from Ayr. A high variation in metal concentrations in the Gladstone diseased group of crabs compared to all other groups suggests that some of these crabs are unable to regulate their metal concentrations and could indicate some level of stress in this group. Levels of all metals in muscle tissue were below those levels recommended by the current Australia New Zealand Food Standards Code, (2000).

CHAPTER 8. Copper Exposure Experiments

Objective 1: *Through the use of copper exposure trials, investigate exposure to metals (in particular copper) as a possible cause of rust spot lesions.*

8.1 INTRODUCTION

Histology of non-perforated rust spot lesions has shown that these lesions are confined to the endocuticular layer, which is laid down in the post moult phase. Observations of recently moulted crabs have also shown that lesions develop within 7-14 days post moult, during this critical phase of endocuticular production (Chapter 6). As previously stated (Chapter 4), the evidence suggests that the lesions appear due to a disruption in development of this layer rather than due to destruction by a pathogen.

The heavy metal profile has highlighted elevated copper concentrations as a possible factor for association with rust spot lesions. Elevated copper concentrations have been found in Gladstone mud crabs sampled in 1996 (Mortimer 2000), 1999 & 2000 and also from seagrass collected in Port Curtis (Prange 1999). Exposure of crustaceans to sublethal concentrations of copper during the post moult phase has been shown to cause a decrease in the post moult uptake of calcium into the shell, due to competition with copper (Scott-Fordsmand and Depledge 1993). Interference with calcium uptake during this crucial phase of cuticle production could possibly cause the type of histological changes seen in rust spot lesions, by allowing the adhesive epithelium of the muscle attachment to tear away from the upper, partially calcified endocuticle. Forces exerted by the normal contractions of these muscles could also cause the partially calcified endocuticle to rupture between the laminations producing a cavity within the endocuticle. The cavity would remain after calcification is complete (Andersen et al. 2000). This line of reasoning has led us into investigating a possible link between copper exposure and rust spot lesions.

EXPERIMENT 1

Aim:

- ? To determine if cuticular defects could be induced in juvenile mud crabs exposed to chronic, sublethal concentrations of copper.
- ? To determine if post moult carapace calcium uptake can be inhibited by exposure to chronic, sublethal concentrations of copper.

8.2 METHODS

Non-diseased juvenile mud crabs (112) from both Darwin and Bribie Island Aquaculture Centres (males and females, carapace width 30-60mm) were distributed equally into two treatment groups:

a) Control water (natural seawater)

b) Treatment water (natural seawater with the addition of 100 ug/l of copper (2) sulphate anhydrous ($CuSO4$ = 159.60).

Each crab was kept in a separate two-litre glass specimen jar under subdued light. Each container also contained black plastic mesh (climbing apparatus) and all jars were also individually aerated (Figure 8.1). The temperature of the water ranged from 26-28 ?C and was changed three times a week. Crabs were fed twice weekly with either prawns or pilchards, two hours prior to a water change. The water pH, salinity, temperature and dissolved oxygen were monitored weekly. Crabs were checked for moults five times weekly and any crabs that had moulted the previous week were examined for lesions, weighed and measured.

Figure 8.1 Copper exposure trial demonstrating individual crab containers with separate aerators (A) and juvenile mud crab within specimen jar. This particular crab has recently moulted and its shed exuviae is lying on the bottom of the jar (insert B).

Prior to the commencement of the experiment all crabs were examined for the presence of rust spot lesions (as determined according to the previously developed grading system). Four crabs were randomly selected for toxicological analyses. This provided baseline tissue concentrations prior to treatment. Samples of muscle,

hepatopancreas and carapace were analysed for copper concentration and carapace samples were also measured for calcium content via ICP-AES (Inductively Coupled Plasma – Atomic Emission Spectrometry). Water and stock solution was also checked regularly for copper concentration.

At five weeks all crabs were again examined for the presence of rust spot lesions. Tissue samples from four crabs from the control group and six from the treatment group were again analysed for copper and calcium as previously described. At this time the copper concentration in the treatment group was increased to 250 ug/l and the feeding was increased to three times weekly, two hours prior to the water changes, for the remaining six weeks of the experiment. New moults were examined 14 days post moult for carapace lesions.

At completion of the 10-week experiment, all crabs were again examined for lesions with the relevant tissues of a further five crabs from each group being analysed for copper and calcium.

8.3 RESULTS

Five crabs from the control group and three crabs from the treatment group died from complications arising from escaping, aeration failure or moult death syndrome, or otherwise unknown causes.

8.3.1 Lesion development

At the five-week lesion check 28.3% of the control group and 26.4% of the treatment group had developed small grade one rust spot type lesions. There was no difference in the prevalence of disease between the two groups. ($X^2 = 0.00$, df = 1, p = 1.000).
Although there was a trend for a higher prevalence of small grade one rust spot type lesions in the treatment group (34.1%) compared to the control group (26.1%) at the ten-week lesion check, this was not significant at the p=0.05 level ($X^2 = 0.358$, df = 1, p = 0.550).

8.3.2 Toxicology

The results of the analyses of hepatopancreas, muscle and carapace from mud crabs sampled at commencement of experiment, 5 weeks and 10 weeks are reported in Tables 8.1a and 8.1b. In instances where equality of variance could not be achieved, ANOVA was applied on square root transformed data. In two instances equality of variances could not be achieved even using log 10 transformed data, and therefore, non-parametric Kruskall-Wallis ANOVA was applied. Comparison of results using One-way ANOVA and *a posteriori* Tukeys HSD concluded that the only significant differences ($p < 0.05$) between groups were: @ 100ug/l; carapace copper, treatment greater than control or baseline (df = 2, F = 7.207, p = 0.01); muscle calcium, treatment and control greater than baseline (df = 2, F = 5.084, p = 0.027); @ 250ug/l; hepatopancreas copper (square root transformed), treatment greater than control and baseline (df = 2, F = 21.144, p = 0.0001); muscle copper, treatment greater than baseline but not control (df = 2, F = 5.960, p = 0.018); carapace copper Kruskall-Wallace), treatment greater than baseline or control ($X^2 = 6.818$, df = 1, p = 0.009).

Table 8.1a Results of tissue analyses of juvenile mud crabs either prior to experiment (baseline), controls (no treatment for 5 weeks) or treatment (exposed to 100ug/l of copper for 5 weeks).

Copper exposure trial @ 100ug/l				
	Group		Mean	Std. Error
Copper hepatopancreas	baseline	Mean	72.88	22.42
ug/g wet wgt.	5 week Control	Mean	77.48	15.57
ns	5 week Treatment	Mean	92.77	17.47
Copper muscle	baseline	Mean	4.73	1.22
ug/g wet wgt.	5 week Control	Mean	4.58	1.21
ns	5 week Treatment	Mean	6.15	1.07
Copper carapace	baseline	**Mean**[b*]	4.33	0.42
ug/g wet wgt.	5 week Control	**Mean**[b]	3.83	0.49
	5 week Treatment	**Mean**[a]	12.68	2.51
Calcium hepatopancreas	baseline	Mean	7903.55	2107.30
ug/g wet wgt.	5 week Control	Mean	1448.26	377.92
ns	5 week Treatment	Mean	7975.09	2133.36
Calcium muscle	baseline	**Mean**[a]	5058.59	1121.18
ug/g wet wgt.	5 week Control	**Mean**[b]	2702.30	377.59
	5 week Treatment	**Mean**[b]	2543.58	248.15
Calcium carapace	baseline	Mean	197.20	9.63
mg/g wet wgt.	5 week Control	Mean	171.75	9.46
ns	5 week Treatment	Mean	177.55	5.06

*Means followed by the same letter are not statistically different ($p<0.05$).
ns = not significant

Table 8.1b Results of tissue analyses of juvenile mud crabs either prior to experiment (baseline), controls (no treatment for 10 weeks) or treatment (exposed to100ug/l for 5 weeks then 250ug/l of copper for 5 weeks).

Copper exposure trial @ 250ug/l				
	Group		Mean	Std. Error
Hepatopancreas copper	Baseline	**Mean**[b*]	8.146	1.47
square root transformed	10 week Control	**Mean**[b]	6.002	1.974
	10 week Treatment	**Mean**[a]	21.036	1.796
Muscle copper	Baseline	**Mean**[a]	4.73	1.22
	10 week Control	**Mean**[ab]	8.02	1.24
	10 week Treatment	**Mean**[b]	11.02	1.28
Hepatopancreas calcium	Baseline	Mean	7903.55	2107.30
ns	10 week Control	Mean	7942.60	1207.81
	10 week Treatment	Mean	9049.81	1532.57
Muscle calcium	Baseline	Mean	5058.59	1121.18
ns	10 week Control	Mean	3764.97	538.05
	10 week Treatment	Mean	2828.87	509.80
	Group		Mean	Std. Dev.
Carapace calcium	Baseline	Mean	197.20	19.254
Non-parametric	10 week Control	Mean	198.90	6.167
ns	10 week Treatment	Mean	235.44	129.42
Carapace copper	Baseline	**Mean**[b]	4.33	0.838
Non-parametric	10 week Control	**Mean**[b]	3.28	0.841
	10 week Treatment	**Mean**[a]	14.18	8.024

*Means followed by the same letter are not statistically different ($p<0.05$).
ns = not significant

8.4 DISCUSSION

8.4.1 Lesion development

Some of the crabs used in the trials were selected from a batches of pond reared crabs obtained from aquaculture centres. Over one third of some batches of crabs could not be used in the trial due to preexisting classic shell lesions. Pathology of a number of these crabs revealed that the likely cause of the lesions was bacterial (Chapter 6). Bacterial shell disease is common in impounded crustaceans and is thought to be facilitated by environmental stressors, such as overcrowding and poor water quality. Prince et al. (1993) noted that as many as 25% of impounded lobsters can be affected.

Therefore it is not surprising that a large number of crabs from both the treatment and control groups developed shell lesions. Although a larger number of crabs in the 250ug/l treatment group developed lesions, there was no significant difference in the prevalence of shell lesions between the control and treatment groups. Most of the crabs moulted at least twice during the course of the experiment. Some of the crabs which developed lesions post moult would go on to shed these lesions on the successive moult and remain free of lesions. Some crabs, however, redeveloped new lesions. None of the lesions were severe, being classed as pin point Grade one lesions.

Juvenile mud crabs were selected for the trial due to their high moulting frequency compared to adults. Subsequent examination of 65 wild caught juvenile mud crabs (Chapter 3) determined that none of the crabs had shell lesions. It is possible that rust spot shell lesions are only restricted to adult populations and could account for the lack of a significant difference in shell disease between our control and treatment groups. These experiments preclude the possibility that other contaminants in conjunction with copper may act synergistically to cause shell lesions.

8.4.2 Toxicology

It is not surprising that tissue concentrations of copper were higher in some of the treatment groups compared to controls, particularly in the 250 ug/l groups as mud crabs are known to accumulate metals from the environment. One of the aims of the experiment was to demonstrate that copper could inhibit calcium uptake in post moult crabs. This was not demonstrated in this experiment. The crabs sampled in this experiment for carapace calcium, however, were hard-shelled crabs in the intermoult phase. It is possible that although there was competition or inhibition/interference by copper on the calcium uptake mechanisms therefore affecting the rate of uptake, the total quantity of calcium required to completely harden the shell was attained by the time the crab reached the intermoult stage. As some questions were also raised as to the accuracy of some of the analyses, it was decided to repeat the copper exposure trial on a smaller scale, sampling crabs for carapace calcium while they were still in the soft post moult phase.

EXPERIMENT 2

Aim:

? To determine if post moult carapace calcium uptake in soft shelled mud crabs can be inhibited by exposure to chronic, sublethal concentrations of copper.

8.5 METHODS

8.5.1 Exposure trial

Non-diseased juvenile mud crabs (24) from Gladstone were harvested from wild stocks using standard mesh, opera house style bait pots. Both males and females, carapace width 44-105mm were distributed into the two treatment groups:

a) Control water (natural seawater)

b) Treatment water (natural seawater with the addition of 250 ug/l of copper sulphate anhydrous ($CuSO4$ = 159.60M).

Each crab was kept in a separate five-litre glass specimen jar under subdued light, similar to that shown in figure 8.1. All jars were individually aerated as for Experiment 1. The temperature of the water ranged from 26-28 ?C and the water was changed three times a week. Crabs were fed three times weekly with either prawns or pilchards, the evening prior to a water change. The water pH, salinity, temperature and dissolved oxygen were monitored weekly.

Crabs were checked at least daily to see if they had moulted and the approximate time of completion of the moult recorded. The exuviae was removed as soon as possible after the moult to prevent ingestion by the crabs. At approximately 72hrs post moult soft-shelled crabs were removed from their jars, killed by freezing (-20°C) and stored frozen for subsequent dissection. Samples of hepatopancreas and carapace from seven post moult control crabs and seven post moult treatment crabs, were analysed for Cu and Ca using ICP-MS. The method of metal analyses was as described in Chapter 7. For calcium analyses, dry matter (at 105 °C) and ashing (at 600 °C) was done by TGA-601 (Thermogravimetric analyser from LECO Australia P/L). Ashing is a minimum of 2 hours to a constant weight, to a maximum of 4 hours. Variation on constant weight is 0.1%. After ashing, samples were digested by concentrated HCl. Samples were further diluted in KCl to prevent interference caused by ionisation of calcium. Calcium was measured by atomic absorption flame spectroscopy (AAS) using a nitrous oxide-acetylene flame (A.O.A.C.), on a Varian Spectra AA 220FS. (Official Methods of Analysis of the association of Official Analytical Chemists (A.O.A.C.). 14th Edition (Washington): Section 7.099 (a)).

8.5.2 Statistical treatment of results: t tests

The aim of these analyses was to test for differences in Ca in Carapace and Cu in hepatopancreas between Control (C) and Test (T) samples. In the first instance, equality of sample variances was tested prior to analysis. Sample variances were shown to be equal on untransformed data - therefore no data transformation was

necessary and parametric tests were applied. Comparison of concentrations between groups (Control and Treatment) was made using two-sample t-tests. The hypothesis being tested was that exposure to elevated Cu would result in reduced Ca in carapace, indicative of inhibition of Ca uptake. T-tests to compare Cu in tissues (hepatopancreas and carapace) would confirm that the experimental exposure to Cu was effective, in that it affected tissue Cu burdens.

T-tests to compare Ca in carapace will test the hypothesis that the exposure to Cu reduces carapace Ca, presumably by inhibition. Comparison of Ca in hepatopancreas as a secondary test will show whether Cu exposure also affects Ca concentrations in hepatopancreas, although this is secondary to the main hypothesis of the experiment.

8.5.3 Statistical treatment of results: ANCOVA

Although all experimental crabs were exposed to the same nominal concentration of Cu, there was a wide range in tissue Cu concentrations (218 – 514 mg/kg), and in some instances hepatopancreas Cu concentrations in Control crabs were higher than in Treatment crabs. To allow for this variation, Cu was used as a covariate in an ANOVA to test for differences in Ca concentration in carapace between Control and Treatment. This analysis standardises for Cu concentration across crabs and then tests for differences in Ca concentration (i.e. do control crabs with a high hepatopancreas Cu concentration have a high or low carapace Ca concentration compared to Treatment crabs with a low hepatopancreas Cu concentration).

8.5.4 Statistical treatment of results: Correlation

Because there is overlap in hepatopancreas Cu concentrations between groups, an alternative way of looking at these data was to ignore the Control and Treatment groups and look for a response between hepatopancreas Cu and carapace Ca by correlation. If a significant relationships existed it might be possible to test for this dependence and describe it statistically using Linear Regression. In the first instance, Pearson Product Moment Correlation and Spearman Rank Correlation were used to test if Cu and Ca in hepatopancreas and carapace varied together.

8.5.5 Statistical treatment of results: Regression

Correlation does not imply cause and effect. Therefore regression was used to determine the extent to which one quantity varied with the other. However, the regression coefficient and significance levels would be the same as for the Pearson correlations above and therefore, in this instance regression was only worth applying to those Pearson correlations that were significant.

8.6 RESULTS

8.6.1 Results: t-tests

Results show that there were significant differences in Cu concentrations between control and treatment groups, with higher concentrations of Cu in Treatment crabs

compared to Control crabs for both hepatopancreas and carapace. This infers that crabs accumulate copper from the surrounding exposure water and that the exposure to Cu was effective (significant results below in shown in bold).

Two Sample t-test for the Means of Carapace Cu between Groups

Group	N	Mean	Std. Dev.	Std. Error
Cont	7	9.971429	3.9487	1.4925
Treat	7	14.85714	3.4976	1.3219

If Variances Are	t statistic	Df	Probability > t
Equal	-2.451	12	**0.0306**

Two Sample t-test for the Means of Hepatopancreas Cu between Groups

Group	N	Mean	Std. Dev.	Std. Error
Cont	7	187.1	87.363	33.02
Treat	7	335.8571	110.3	41.688

If Variances Are	t statistic	Df	Probability > t
Equal	-2.797	12	**0.0161**

Two Sample t-test for the Means of Carapace Ca between Groups

Group	N	Mean	Std. Dev.	Std. Error
Cont	7	20.17143	2.6581	1.0047
Treat	7	16.01429	1.5486	0.5853

If Variances Are	t statistic	Df	Probability > t
Equal	3.575	12	**0.0038**

Two Sample t-test for the Means of Hepatopancreas Ca between Groups

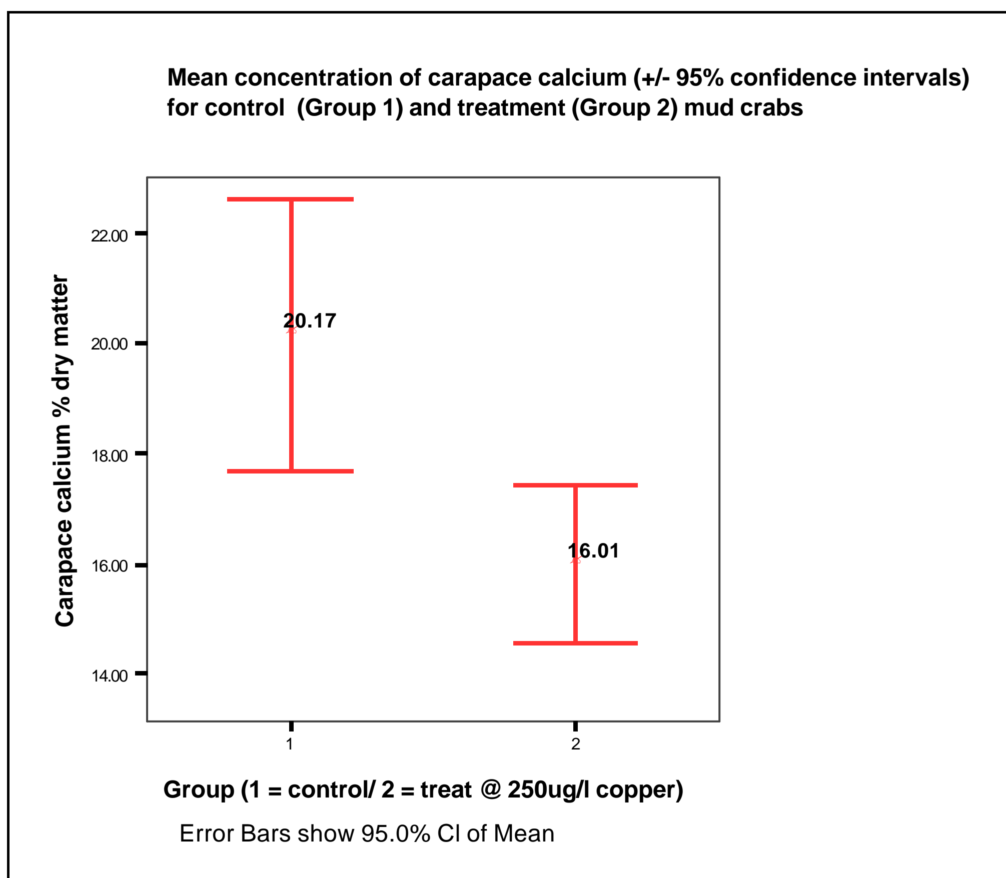
Group	N	Mean	Std. Dev.	Std. Error
Cont	7	2.857143	1.9924	0.753
Treat	5	2.08	1.0354	0.463

If Variances Are	t statistic	Df	Probability > t
Equal	0.792	10	0.4469

The T-tests to test the main hypothesis of the experiment indicate a higher concentration of Ca in the carapace of Control crabs compared to Treatment Crabs (Figure 8.2). This infers that elevated Cu in the exposure water inhibits uptake of Ca into the carapace. There was no significant difference in Ca concentration of hepatopancreas between groups suggesting that Cu does not affect Ca concentrations

in hepatopancreas (NB there was a trend of higher Ca in hepatopancreas of Control versus Treatment crabs which suggests there may be some regulation by Cu; 2.86 versus 2.08 mg/kg Ca).

Figure 8.2 Mean concentration of carapace calcium (=/- 95% confidence intervals) for control (Group 1) and treatment (Group 2 – exposed to chronic sublethal concentrations of 250ug/l of copper) mud crabs



8.6.2 Results: ANCOVA

The ANOVA with hepatopancreas Cu concentrations used as a covariate indicated there was still a significant difference in carapace Ca ($p = 0.0057$), again supporting the hypothesis.

8.6.3 Results: Correlation

Pearson Product Moment Correlation Coefficients giving correlation coefficient, significance level and number of observations

	CarapCu	CarapCa	HepCa	Hepcu
CarapCu	1.00000			
CarapCu	-			
	14			
CarapCa	-0.13833	1.00000		
CarapCa	0.6372	-		
	14	14		
HepCa	-0.25695	-0.28470	1.00000	
HepCa	0.4201	0.3698	-	
	12	12	12	
Hepcu	0.51226	-0.47405	-0.10332	1.00000
Hepcu	**0.0611**	**0.0868**	0.7493	
	14	14	12	14

Spearman Rank Correlation Coefficients giving correlation coefficient, significance level and number of observations

	CarapCu	CarapCa	HepCa	Hepcu
CarapCu	1.00000			
CarapCu	-			
	14			
CarapCa	-0.18605	1.00000		
CarapCa	0.5242	-		
	14	14		
HepCa	-0.02124	-0.32805	1.00000	
HepCa	0.9478	0.2979	-	
	12	12	12	
Hepcu	0.54426	-0.51705	0.05634	1.00000
Hepcu	**0.0442**	**0.0583**	0.8619	-
	14	14	12	14

Although there was no significant positive correlation ($p < 0.05$) there was a trend (Pearson and Spearman at ? = 0.1) in Cu concentrations between carapace and hepatopancreas, indicating concurrent increases in concentrations of Cu in both tissues. There was also a trend in the correlation (Pearson and Spearman at ? = 0.1) between Ca in carapace and Cu in hepatopancreas, indicating a decline in Ca concentrations in the carapace as Cu concentrations in the hepatopancreas increase. There was no relationship between carapace Cu and carapace Ca concentrations.

8.6.4 Results: Regression

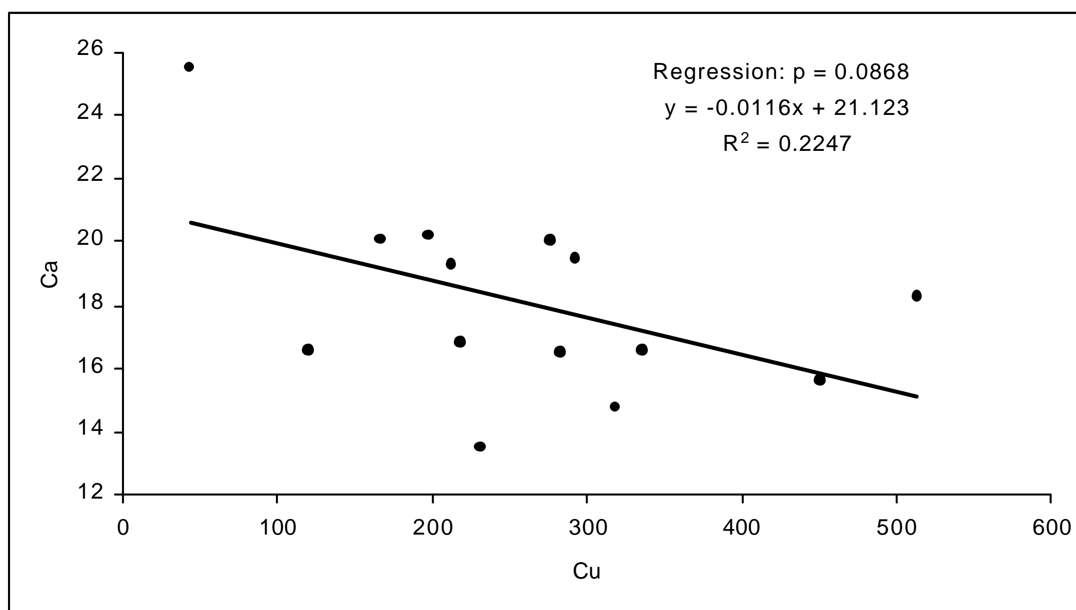
Linear Regression was not significant ($p < 0.05$) but at ? = 0.1. the regression explained approx 16% of variation in the data. Therefore, although there is a trend (i.e. a vague relationship between Cu in hepatopancreas and Ca in carapace), the relationship is not strong (Figure 8.3).

Regression of Ca as dependent variable against Cu as causal factor.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	26.35316	26.35316	3.48	0.0868
Error	12	90.91613	7.57634		
Corrected Total	13	117.26929			

Adj R-Sq = 0.1601 (i.e. the regression equation explains on 16% of the variation in Cu and Ca data – a very poor cause & effect relationship).

Figure 8.3 Regression of Cu in hepatopancreas against Ca in carapace



8.7 DISCUSSION

Experiment 2 confirmed that calcium uptake into the carapace of soft-shelled crabs (72 hours post moult) was inhibited by sublethal copper exposure. The data show that when juvenile mud crabs are exposed to Cu they uptake the metal into hepatopancreas and carapace tissues. There is also significantly less Ca in carapace of treatment crabs compared to control crabs supporting inhibition of Ca uptake by Cu exposure. Ca inhibition appears strongest in carapace, with only a trend of inhibition in hepatopancreas. The significance of the test for hepatopancreas, however, may have been affected by the smaller sample size due to insufficient sample for analyses (5 versus 7 replicates). It is interesting to note that the uptake concentrations (i.e. hepatopancreas Cu concentrations) vary quite considerably (218 – 514 mg/kg) and actually overlap with Cu concentrations in control crabs. It is also interesting to note a relatively wide range in hepatopancreas Cu concentrations in control crabs, similar to the large variability in metal concentrations experienced in adult Gladstone diseased crabs discussed in Chapter 7. As the juvenile crabs were harvested from wild stocks from Port Curtis and not depurated prior to the experiment, it is not surprising to see a high variation in copper concentrations among this group, similar to that found in the adult crabs.

Other metals have also been known to cause interference with calcium uptake in aquatic organisms by inhibition, competition or interference with cell membrane active transport mechanisms (Verbost et al 1992) and have also been shown to cause shell disease (Doughtie et al 1983). It is likely that copper and possibly other metals are implicated in the aetiology of the lesions we have studied. At ecdysis, approximately 90% of the whole body calcium is lost with the exuviae (Scott-Fordsmand and Depledge 1997). In adult crabs, relatively little calcium e.g. less than 20% is stored between moults in an amorphous form in regions of the gut (Wheatly 1999). Endocuticular deposition begins after the moult and its calcification occurs simultaneously with the formation of the organic lamellae (Roer 1980). Shortly after the moult, there is also a rapid increase to a high rate of calcium flux across the gill and carapace epithelium (Neufeld and Cameron 1993).

Exposure of *Carcinas maenas* to sublethal concentrations of copper during the post moult phase has been shown to cause a decrease in the carapace calcium content of paper shell crabs, possibly due to a reduced uptake of calcium from the environment (Scott-Fordsmand and Depledge 1993). Bjerregaard and Vislie (1986) suggested that copper could inhibit active transport of calcium from haemolymph to exoskeleton, by inhibiting the Ca-ATPase or Na/Ca exchange pump. Other metals have been shown to inhibit calcium and ion uptake in aquatic organisms due to competition/interference at common binding sites at gill surfaces (Verbost et al. 1992, Wright 1995, Harris and Santos 2000). Weinstein et al. (1992) in their study of shell disease and metal contents of blue crabs (*Callinectes* sapidus) commented that “interference with the normal process of calcification during the formation of the cuticle in crabs could produce a structurally weakened shell more vulnerable to injury and thus more susceptible” to shell disease.

Therefore, the inhibition of calcium uptake into the post moult carapace by copper would appear to be a contributing factor in the development of rust spot lesions in Gladstone crabs. Although copper is potentially one of the most hazardous metals present in the marine environment (Langston 1990, Hebel et al. 1997), an effect due to the combined impact of several heavy metals and perhaps other contaminants is more likely to be implicated in the aetiology of rust spot shell disease.

Conclusion

Through the use of copper exposure trials in which juvenile mud crabs were exposed to sublethal concentrations of copper, we explored the hypothesis that copper exposure inhibits calcium uptake into the post moult crab shell. The trial confirmed that calcium uptake into the carapace of soft-shelled crabs (72 hours post moult) was inhibited by sublethal copper exposure. There was a significant negative relationship between increasing copper concentrations in the hepatopancreas and declining calcium concentrations in the carapace. Other metals including copper have been shown to cause interference with calcium uptake in crustaceans. It is therefore conceivable that exposure to copper, perhaps in combination with other metals/contaminants could be implicated in the cause of rust spot shell disease.

CHAPTER 9. Biochemical Parameters

Objective 1: *Define the histological stages of the lesions by developing a pathological sequence of events (includes pathology tests).*

9.1 INTRODUCTION

Fluctuations in the concentrations of haemolymph immune factors can occur in aquatic organisms due to exposure to a range of external factors. Changes in the immune system can occur not only due to challenge by pathogens, but also from exposure to environmental contaminants (O'Halloran et al. 1998, Lowe and Fossato 2000). Therefore a number of different measures of immune responses have been developed by researchers, as biomarkers of environmental health in aquatic systems (O'Halloran et al. 1998).

A range of haemolymph assay tests was carried out in an effort to determine the immune status and blood enzyme levels of Gladstone diseased and non-diseased crabs in comparison to their non-diseased counterparts in Ayr. Over 300 haemolymph samples were collected for use in pilot trials to develop a suitable multi-assay approach. This included an antibactericidal test, a phenoloxidase test (PO), a glutamine dehydrogenase (GLDH) assay (a cellular enzyme rather than an immune parameter), a red blood cell (RBC) agglutination test and vitamin A and E assays. However, only the first three tests were developed to give consistent, repeatable results and are presented in this report.

Antibacterial activity (Noga et al. 1996c) and phenoloxidase activity (Johansson and Soderhall 1989; Chisholm and Smith 1992) appears to be confined to the haemocytes. Antibacterial activity has been identified in many invertebrates including *Scylla serrata* (Chattopadhyay and Chatterjee 1997) and depressed levels of antibacterial activity have been shown to coincide with an increased prevalence of shell disease in blue crabs *Callinectes sappidus* (Noga et al. 1994, 1996b).

The prophenoloxidase activating system (proPO) is an enzymatic cascade associated with pathogen defence mechanisms in invertebrates (Cardenas and Dankert 1997). The enzyme phenoloxidase is the terminal component produced by the proPO system (Sung et al 1998) and is responsible for the production of melanin in the cuticle after wounding (Aspan et al. 1995). It has been isolated from a number of crustaceans including the shore crab *Carcinus maenas* (Chisholm and Smith 1992).

GLDH is cell enzyme used to detect acute/toxic liver damage in veterinary medicine (Cornelius 1980). This enzyme has also been reported in hepatopancreas and muscle tissue from *Scylla serrata* (Reddy and Bhagyalakshmi 1994).

9.2 METHODS

9.2.1 Collection of specimens

Crabs were obtained live from commercial fisherman and examined. Examination included sexing, measuring the carapace width (to the nearest 5mm) with a standard metric ruler and recording details of any carapace lesions. Fifteen diseased (DS) female mud crabs from Gladstone, each with at least one large, grade two (non-perforated) rust spot lesion, were selected. Due to unavailability, no diseased male crabs were used in this comparison. Non-diseased (ND) mud crabs from Gladstone (GS) (fifteen females and fifteen males) and Ayr (CT) (fifteen females and fifteen males) were also collected for comparison. These were the same crabs from which tissues were collected for the 2000 metal analyses described in Chapter 7.

Diseased crabs were graded according to the method described previously in Chapter 3. Haemolymph (5 to 8 ml) was collected aseptically from the anterior aspect of the proximal arthrodial membrane of the 5th periopod of crabs chilled at 6ºC for 1 hour (Figure 9.1). The haemolymph was kept frozen (-20ºC to -80ºC) until required for testing. Each sample was homogenised briefly, sonicated, centrifuged at 50,000g for 30 minutes and the haemocyte lysate (HLS) extracted for analyses, according to a modified method (Noga *et al* 1994, 1996c).

Figure 9.1 Collection of haemolymph from mud crabs for immune parameters.

9.2.2 Haemolymph antibacterial test

Rows B to H of a 96 well-chilled micro titre plate were each filled with 50?1 of phosphate buffered saline containing 1% salt (PBS). To the first row of wells, 100?1 of serum was added and serially diluted down the plate. Trypticase soy broth containing 1% salt was used to grow up a local *Vibrio sp.* of bacteria. A few colonies were added to 3ml of broth and left at room temperature for 2 or more hours until turbidity equalled 0.5 on the MacFarland Standard. This culture was swabbed onto

trypticase soy agar containing 1% salt (Acumedia Manufacturers Inc.). One 10?1 sample from each of the 8 wells of one serially diluted serum sample was immediately placed onto each of two swabbed agar plates to give a duplicate set of results for each dilution for each crab. A 10?1 sample of PBS was used as a negative control while a 10?1 sample of a serum of known titre was used as a positive control. Plates were incubated at 25°C for 18 to 24 hours. Clear zones indicated inhibition of bacterial growth. Results were recorded as the reciprocal of the highest dilution, which produced any clearing of the *Vibrio sp.* colonies. The General Linear Models (GLM) procedure in the SAS statistical package was used to perform two-way analysis of variance (ANOVA) to test for between-group and between-crab differences in Antibacterial activity (the mean of the two data sets was used). Prior to analysis, data were log. (x+1) transformed to achieve normality and equality in variances. Tukey's Multiple Range test was applied to locate between-group differences where there was a significant main effect.

9.2.3 Phenoloxidase test

Haemolymph was assayed for phenoloxidase (PO) by a modification of the method of Perazzolo and Barracco (1997). Haemolymph was diluted 1:3 with 0.2 M sodium phosphate buffer pH 7.6. Phenoloxidase activity was determined as follows: Replicates of 50?1 of haemolymph were mixed with 50?1 of 1 mg/ml trypsin in buffer or buffer only together with 50?1 of 3 mg/ml of L-DOPA (L-3, 4-Dihydroxyphenylalanine). Samples were incubated for 60 minutes at room temperature and the absorbance at 490 nm recorded. PO activity was expressed as the change in absorbance per minute per ml of haemolymph tested. One-way ANOVA was applied to results and a table of ranked means established.

9.2.4 Glutamate dehydrogenase assay

Freshly collected haemolymph was diluted 1:3 with an anticoagulant (Balaji et al 1989) and analysed for glutamate dehydrogenase (GLDH) by a glutamate dehydrogenase assay kit (Radox Labs. U.K.). One-way ANOVA was applied to results and a table of ranked means established.

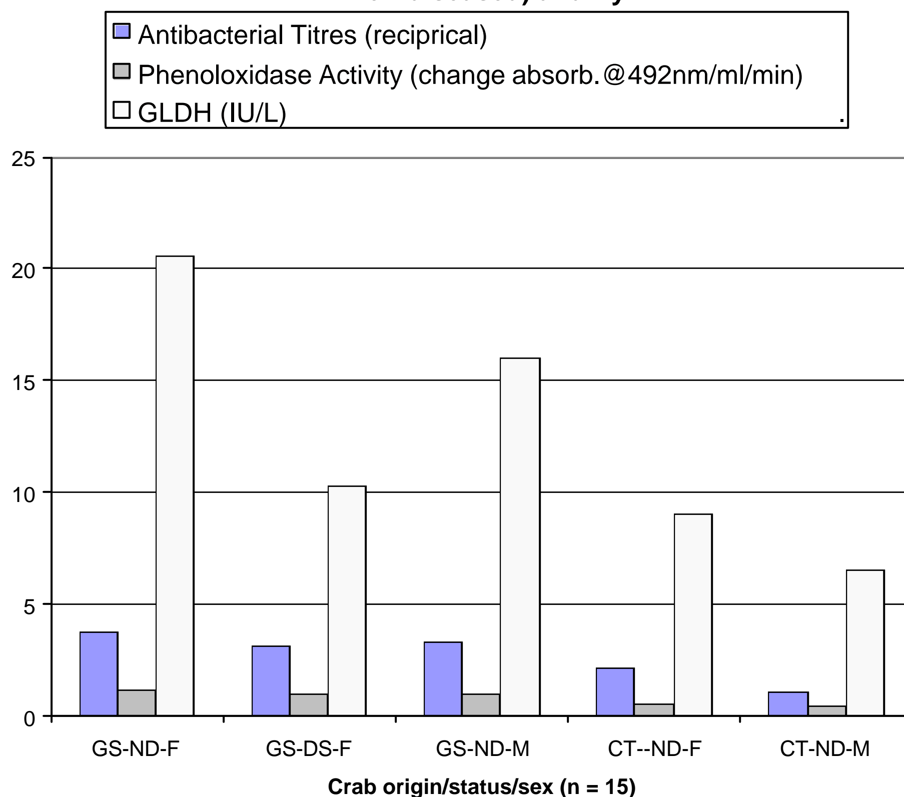
9.3 RESULTS

Results of immune tests are presented in Table 9.1

9.3.1 Haemolymph antibacterial test

Both the non-diseased female and male crabs from Gladstone had higher antibacterial titres than those of the same sex from Ayr. Non-diseased female crabs from both Ayr and Gladstone had higher antibacterial titres than the non-diseased male crabs from their respective areas. However, for the female crabs from Gladstone, the diseased females had lower titres than did the non-diseased females (df = 4, 74, F = 27.67, p <0.0001).

Table 9.1 Tabulated means of antibacterial assay, phenoloxidase test and GLDH assay of Gladstone and Ayr mud crabs.



Legend: GS-ND-F Gladstone non-diseased females
GS-DS-F Gladstone diseased females
GS-ND-M Gladstone non diseased males
CT-ND-F Ayr non-diseased females
CT-ND-M Ayr non-diseased males

Data table: Means in each test followed by the same superscript letter are not significantly different at the 5% level of significance.

Test type	GS-ND-F	GS-DS-F	GS-ND-M	CT—ND-F	CT-ND-M
Antibacterial Titres (reciprocal)	3.733 [a]	3.103 [b]	3.306 [b]	2.118 [c]	1.091 [d]
Phenoloxidase Activity (change in absorb. @ 492nm/ml/min)	1.15 [a]	0.98 [b]	0.99 [b]	0.57 [c]	0.41 [d]
GLDH (IU/L)	20.6 [a]	10.33 [b]	16.0 [a]	9.0 [b]	6.53 [b]

9.3.2 Phenoloxidase assay

All the Gladstone crabs had higher concentrations of PO than either group of crabs from Ayr. In addition, the non-diseased female crabs at each site had a higher concentration of PO than did the non-diseased males from each site. However, of the female Gladstone crabs, the diseased crabs had a lower concentration of PO than did the non-diseased crabs (df = 4, 74, F = 34.77, $p < 0.001$).

9.3.3 Glutamate dehydrogenase assay

Both the female and male non-diseased crabs from Gladstone had higher concentrations of GLDH than those crabs of the same sex from Ayr. Non-diseased female crabs from both Ayr and Gladstone had higher concentrations of GLDH than the non-diseased male crabs from their respective areas although the difference was not significant. For the female crabs from Gladstone, the diseased crabs had lower concentrations of GLDH than did the non-diseased crabs (df = 4, 74, F = 10.03, $p < 0.001$).

9.4 DISCUSSION

The results of elevated haemolymph antibacterial titres in Gladstone crabs emulates that of the phenoloxidase enzyme assays and suggests that this group of crabs has been subjected to a higher degree of stress than have the Ayr crabs and hence have been stimulated to produce higher levels of these immune factors. Alterations in immune levels in aquatic organisms can occur due to exposure to pathogens (Evans et al. 1968, Moullac et al 1997) as well as stress. Ueda et al. (1999) reported a reduction in the antibacterial titre in both the swimming crab (*Ovalipes punctatus*) and the kuruma prawn (*Penaeus japonicus*) when they were stressed by being placed in sawdust and transported for 8 hours. Several authors have also demonstrated immunmodulation in aquatic organisms due to exposure to contaminants (Smith et al. 1995, Reddy 1997, Dyrynda et al. 1998, Dethloff and Bailey 1998, Pipe et al. 1999, Sanchez-Dardon et al.1999).

For the female crabs from Gladstone, the reduced antibacterial titres and PO levels in the diseased crabs compared to those in the non-diseased crabs could suggest that the production of these factors has been suppressed and/or the factors have been made unavailable and/or have been destroyed at a faster rate than normal. These results were similar to the findings of Noga et al. (1994) who recorded reduced concentrations of antibacterial factors from blue crabs (*Callinectes sapidus*) with shell disease from estuarine areas with increased pollution, compared to non-diseased crabs from the same location. The antibacterial activity of both these groups (Noga et al. 1994), however, was also significantly lower than crabs from more oceanic control sites. These latter findings are, however, in contrast to those in our mud crabs. Although the antibacterial titres of diseased female mud crabs were significantly lower than those of the non-diseased female crabs from Gladstone, they were still significantly higher than the non-diseased female crabs from Ayr. The unique histology of our mud crab shell disease in comparison to that of blue crab shell disease and the likely differences in aetiology and epidemiology of the two diseases might explain the contrasting results.

The raised antibacterial titres and PO levels in non-diseased female crabs from both Ayr and Gladstone compared to the males would seem to indicate that females

normally have higher levels than males. Radhika et al. (1998) recorded similar results in that the PO activity in male fairy shrimp, *Streptocephalus dichotomus* was only one-third of that measured in female shrimp. The elevated GLDH values recorded in female non-diseased crabs when compared to male non-diseased crabs although not statistically different, suggest that female crabs might normally have higher levels of tissue damage than males also.

The elevated values of GLDH obtained from both female and male non-diseased Gladstone crabs compared to Ayr crabs, indicate that these crabs had experienced increased stress and tissue damage. Although it is unknown why GLDH levels obtained from the diseased Gladstone female crabs were reduced compared to the non-diseased Gladstone female crabs, it may indicate a reduction in the production, release and/or availability and/or an increased rate of destruction/disposal of this enzyme once released. When Reddy and Bhagyyalakshmi (1994) exposed *Scylla serrata* to a sublethal concentration of 2.5 ppm of cadmium chloride for 96 hours prior to samples being taken, they recorded an increase in GLDH, which indicated, increased tissue damage. Gladstone mud crabs, however, have elevated levels of a number of different metals suggesting chronic rather than acute exposure to these metals. Although the effect of chronic exposure to metals on GLDH levels is unknown, it is possible that in diseased female crabs from Gladstone, the metals may have "tied up" many of the cellular enzymes (Seawright 1989) including GLDH, rather than having caused acute necrosis. Adaptive mechanisms of immune responses to chronic exposure to metals have been observed (Harris and Santos 2000) and may have an effect on enzyme levels. Zelikoff et al. (1995) also noted that it is also common to observe an enhanced immune response at low concentrations of heavy metals, followed by suppression at higher exposure levels.

Although perturbations of immune functions caused by a range of immunotoxic compounds can lead to immunosuppression (Smith and Johnston 1992, Sanchez-Dardon et al. 1999, Zhang et al. 2000), immune responses can also become enhanced in response to foreign substances (Reddy 1997, Pipe et al. 1999, Cima et al. 1999, Fatima et al 2000). Results are therefore varied and indicate that not all immune parameters are affected in the same way by contamination and that the type and extent of effects vary not only with the nature or concentration of the contaminants but with temporal variations (Dyrynda et al. 1998) and physiological differences of the organism or species being studied (Harris and Santos 2000).

Conclusion

Results of crab blood tests in which two immune parameters and one cell enzyme were measured, suggest that Gladstone crabs have been stimulated to produce higher levels of these factors compared to the Ayr crabs and could indicate some level of stress in Gladstone crabs. Exposure to pathogens, contaminants and stress is known to alter production of these factors in aquatic organisms. Although the immune factors in the female diseased group of crabs from Gladstone were elevated compared to the female crabs from Ayr, they were significantly lower than levels in the non-diseased female crabs from Gladstone. This suggests that production in this diseased group may have been suppressed or the factors have been destroyed at a faster rate then normal.

CHAPTER 10. Metal Burden Shell Disease and Blood Parameters

10.1 INTRODUCTION

Several authors have attributed some types of shell disease to exposure to pollutants including heavy metals (Doughtie et al. 1983, Feely 1983, Bullis et al. 1988). Other authors have established a relationship between presence of shell disease and a modulated immune response (Noga et al. 1994). Disturbances of the immune system in aquatic organisms can occur not only due to challenge by pathogens, but also from exposure to environmental contaminants (O'Halloran et al. 1998, Lowe and Fossato 2000). Therefore the concept of a cause-effect relationship between metal burdens, shell disease and immune responses of the mud crabs, which we have studied, was one that required further investigation. Although GLDH is a cell enzyme rather than an immune indicator, it will be included in the term immune response in the proceeding chapter.

10.2 METHODS

Stepwise multiple regressions were used to investigate whether any relationships exist between the measures of mud crab immune response (Chapter 9) and the hepatopancreas metal concentrations (Chapter 7) of those same crabs, sampled from Ayr and Gladstone in 2000. This statistical test treats an immune response as the dependent variable, and uses changes in the concentration of one or more metals to best explain changes in immune response. For a metal to enter the regression equation it must have a significant relationship (i.e. $p< 0.05$) with the immune response variable. All metals that are significant and help to explain changes (variability) in the level of an immune response will remain in the final equation. Where there are two closely correlated metals, however, one may enter the regression equation whilst the other will not because the former explains the majority of the variability in the immune response, with the latter providing little additional information. Also the assumption of the analysis is that there is cause and effect i.e. there is a 'process' whereby immune response changes as a function of a change in the concentration of one or more metals. Stepwise multiple regressions will reveal a statistical relationship, but does not prove cause-effect (i.e. they provide no proof of a process); therefore on interpreting the results these assumptions should be considered.

Five measures of immune response were used:

1. Antibacterial Activity Measure #1 (ABR1),
2. Antibacterial Activity Measure #2 (ABR2),
3. Mean of both measures of Antibacterial Activity (mABR),
4. Phenoloxidase Activity (Pheno), and
5. Glutamate Dehydrogenase Activity (GLDH).

(NB. Duplicate measures of antibacterial activity were measured for each specimen)

All available hepatopancreas metal data were used as the independent variables (n = 28 metals). In addition to the individual metals data, total metal burden for each crab was calculated and used as an additional independent variable.

1. All metals for which at least one value out of the 75 samples exceeded detection (> 0.1 mg/kg) were retained for analysis – this left 28 metals with at least one value above detection. Within these metals, if any values were less than detection, half the detection level was taken as the value to be used in analysis (i.e. for a detection level of <0.1, a value of 0.05 was used).

2. Within each metal, the values were standardised to a scale of zero to 1 by the equation: xnew = (x-xmin)/xrange. This provides a relative indication of the burden of each metal in each sample (i.e. samples with a high concentration of a metal will have a value close to 1 and samples with a low concentration will have a value close to zero) (NB the only shortcoming of this standardisation is that it relies on a relative difference in metal concentrations between samples (i.e. the range); if all samples (Ayr and Gladstone) have high concentrations of a metal – then they will all appear to have a low burden).

3. To derive a total burden of all metals in each of the 75 samples, the sum of the individual burdens across the 28 metals was calculated. This provided a total metal burden for each of the 75 samples. (NB. This approach makes no assumptions regarding the differing toxicity of different metals).

All metals (including metal burden) were included in the analyses as untransformed data but also as log (x+1) transformed data, effectively doubling the number of independent variables. Analyses were restricted, however, so that each metal could only appear in any regression equation in only one form (untransformed or transformed), but not both. In the first instance, regressions were performed using untransformed dependent variables (immune response variables), but subsequently analyses were repeated against log (x+1) transformed immune response variables. The use of transformations allows for better solutions if the response between dependent and non-dependent variables is non-linear.

Analyses were preformed on all crabs combined (Ayr and Gladstone, Males and Females, Diseased and Non-diseased) and then on each subset of crabs within the Gladstone and Ayr datasets:

1. All Gladstone and Ayr crabs (Males and Females, Diseased and Non-diseased),
2. All Gladstone crabs (Males and Females, Diseased and Non-diseased),
3. Female Gladstone crabs (Diseased and Non-diseased),
4. Female Gladstone crabs (Diseased),
5. Female Gladstone crabs (Non-diseased),
6. Male Gladstone crabs (Non-diseased),
7. Female Ayr crabs and
8. Male Ayr crabs.

In total, 80 multiple regressions were performed. The r-squared (proportion of variation in the dependent variable (*viz.* immune factor) explained by the metal or metals), and the significance (p-value) of each regression were tabulated. Within each subset of crabs, a multi-fit plot was produced for the best regression solution. This is a plot of the observed changes in the immune response against the predicted based on the changes in the metal or metals used in the final regression equation. If a multi-fit

plot showed that the relationship was effectively a two-point correlation (i.e. two groups of samples widely separated), then the result was ignored.

For the best multi-fit plot within each subset of crabs, the individual plots of each metal included in the final regression equation against the immune response were plotted to illustrate the strength and direction of the individual relationships.

10.3 RESULTS

Results are presented for all crabs combined and then for each subset of crabs within the Gladstone and Ayr datasets as previously outlined. Selected equations are presented as plots in each subset to demonstrate the more significant relationships.

10.3.1 All crabs (Ayr & Gladstone, Male & Female, Diseased and Non-diseased).

Table 10.1. Results of stepwise, multiple regressions of metals (independent), against immune responses (dependent). Results in **bold**, are illustrated in a multi-fit plot (Figure 10.1). The direction of each metal in the final equation is positive, unless the name of the metal is underlined, indicating that the relationships were negative (i.e. immune response increased as the metal concentration decreased). 'Log' indicates that the variable was log (x+1) transformed.

Immune Response	R^2	p-value	Metals in final equation
ABR1	.197	.0005	logCr+logZn
ABR2	.229	.0001	logCr+logCu
mABR	.209	.0003	logCr+logZn
Pheno	**.515**	**<.0001**	**logCr+logCu-logBurden-Ba+Co**
GLDH	.283	<.0001	logBa+Cu
logABR1	.294	<.0001	logCr+logCu
logABR2	.406	<.0001	logBa+logCr+logCu
logmABR	.389	<.0001	logBa+logCr+logCu
logPheno	.467	<.0001	logCr+logCu-Ba+Co
logGLDH	.296	<.0001	logBa+Cu

Although there were some significant relationships between immune responses and metal levels in the combined group of crabs, none of the R^2 values were very high indicating weak relationships. The plot for phenoloxidase, however, is presented below and shows a clear separation between Ayr and Gladstone samples. Some relationships are negative as represented by logBurden (log of total metal burden) in this equation.

10.3.2 All Gladstone crabs (Males & Females, Diseased & Non-diseased)

Results of stepwise multiple regression for Gladstone crabs combined is reported in Table 10.2. There were some relationships identified in the Gladstone group of crabs, however, again the R^2 values were not high. A breakdown into individual crab groups demonstrates more obvious relationships.

Figure 10.1 Observed Phenoloxidase versus predicted phenoloxidase using the equation Pheno = logCr+logCu-logBurden+Ba+Co (R-sqr = 0.515) for All Crabs (Ayr/GS, Female/Male, DS/ND, indicating position of Ayr versus Gladstone crabs and sex/condition of Gladstone crabs.

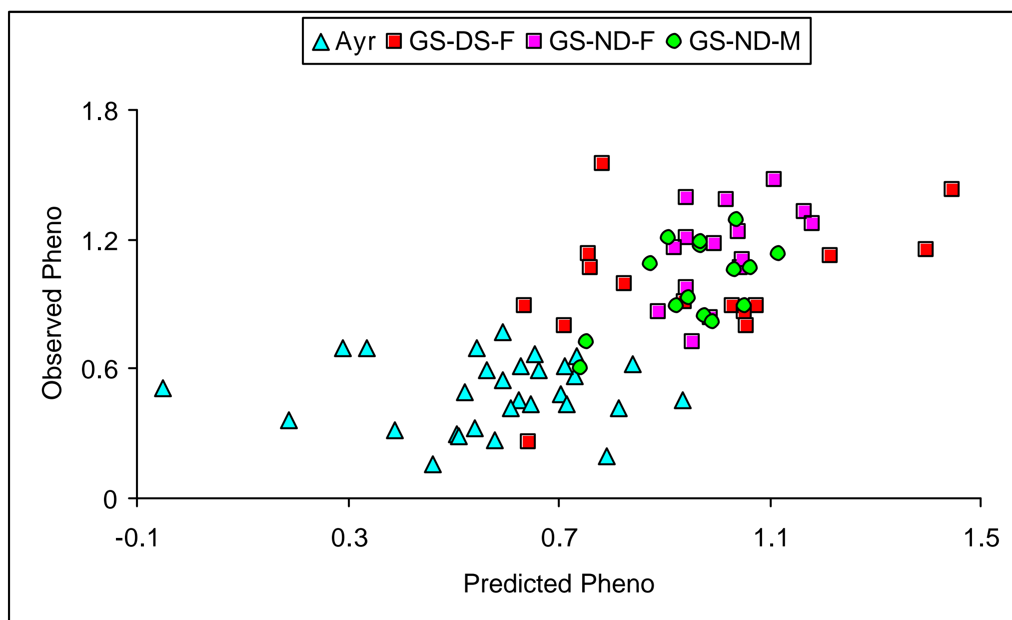


Table 10.2. Results of stepwise multiple regressions of metals (independent) against immune responses (dependent) for all Gladstone crabs (Males & Females, Diseased & Non-diseased). Results in *italics* indicate a two-point regression with a misleadingly high R^2 value. The direction of each metal in the final equation is positive, unless the name of the metal is underlined, indicating that the relationship was negative (i.e. immune response increased as the metal concentration decreased). 'Log' indicates that the variable was log(x+1) transformed.

Immune Response	R^2	p-value	Metals in final equation
ABR1		ns	
ABR2		ns	
mABR		ns	
Pheno	.266	.0008	-logLa+Mo
GLDH	.200	.0021	logCu
logABR1		ns	
logABR2		ns	
logmABR		ns	
logPheno	.308	.0004	-logLa+Mo
logGLDH	*.441*	*<.0001*	*-logSb+Cd-Sr*

10.3.3. All Gladstone female crabs (Diseased & Non-diseased)

Results of regression analyses for Gladstone female crabs combined highlighted only two significant relationships described in Table 10.3. Separate regressions of the female diseased and non-diseased groups, however produced more significant results (Table 10.4, Table 10.5).

Table 10.3. Results of stepwise multiple regression of metals (independent) against immune responses (dependent) for all Gladstone female crabs (Diseased & Non-diseased). The direction of each metal in the final equation is positive, unless the name of the metal is underlined, indicating that the relationship was negative (i.e. immune response increased as the metal concentration decreased). 'Log' indicates that the variable was log(x+1) transformed.

Immune Response	R^2	p-value	Metals in final equation
ABR1		ns	
ABR2		ns	
mABR		ns	
Pheno		ns	
GLDH	.379	.0016	logCu-Sr
logABR1		ns	
logABR2		ns	
logmABR		ns	
logPheno		ns	
logGLDH	.589	.0001	-logSb+Cd+Sr

10.3.4. All Gladstone female Diseased crabs

A number of significant relationships between metal levels and immune responses were identified in the Gladstone female diseased group of crabs. Some of the R^2 values were high indicating strong relationships (Table 10.4). The plot of the equation representing GLDH is presented in Figure 10.2. The equation is explained by three separate metals, whose plots are also presented in Figure 10.3. Copper explains 64% of the relationship (R^2 value of 87%). Interestingly Sr represents a small but negative response.

Table 10.4. Results of stepwise multiple regressions of metals (independent) against immune responses (dependent) for all Gladstone female Diseased crabs. Results in *italics* indicate a two-point regression with a misleadingly high R^2 value. Results in **bold** are illustrated in a multi-fit plot (figure 10.2). The direction of each metal in the final equation is positive, unless the name of the metal is underlined, indicating that the relationship was negative (i.e. immune response increased as the metal concentration decreased). 'Log' indicates that the variable was log(x+1) transformed.

Immune Response	R^2	p-value	Metals in final equation
ABR1	.509	.0028	logCr
ABR2	*.734*	*.0004*	*logCu+Cr*
mABR	*.600*	*.0007*	*Cr*
Pheno		ns	
GLDH	**.873**	**<.0001**	**As+Cu-Sr**
logABR1	.540	.0018	logCr
logABR2	.397	.0118	Cr
logmABR	.506	.0029	logCr
logPheno		ns	
logGLDH	.8614	<.0001	logCu-Sr

Figure 10.2 Multifit plot of GS - DS females, GLDH = As+Cu -Sr, R-sqr = 0.8725. Site equals (EQ) Gladstone (GS) and sex equals female (F) and condition equals diseased (DS).

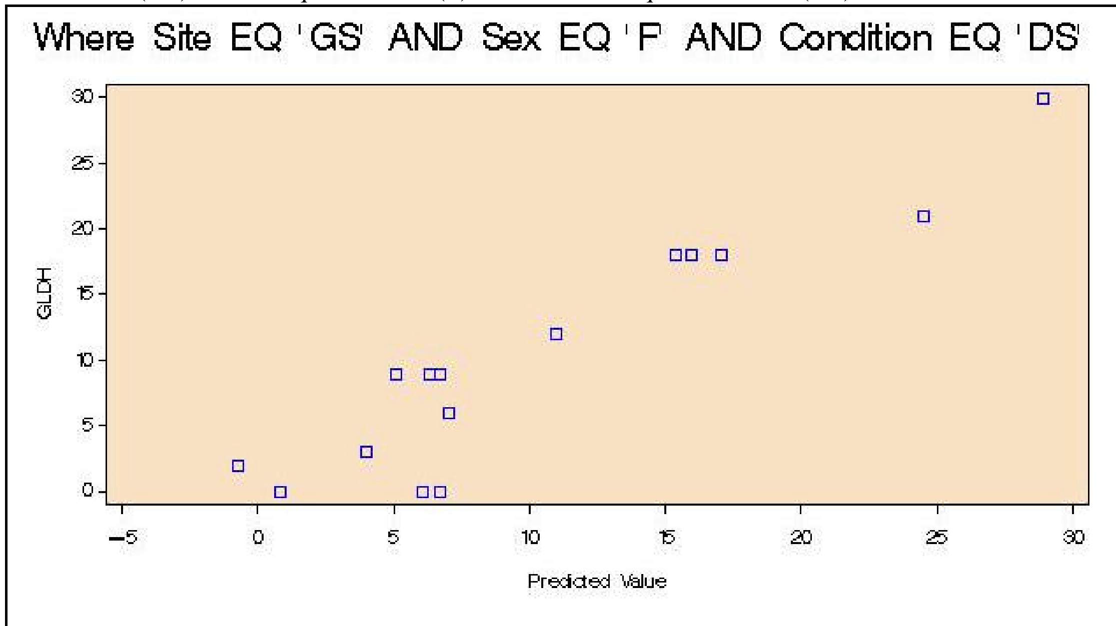
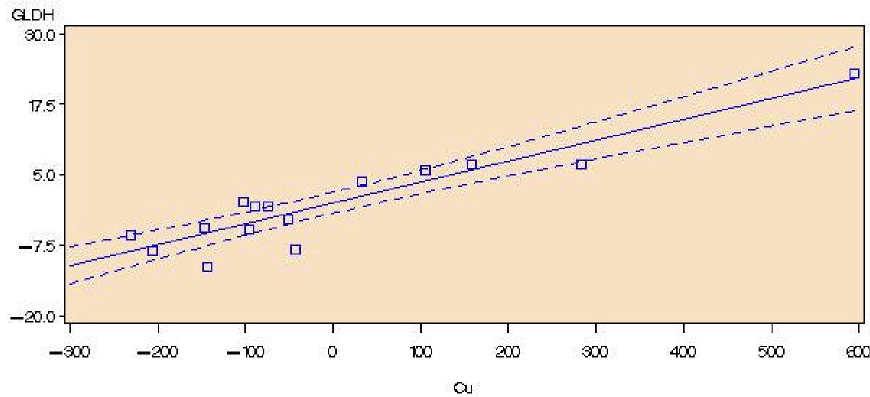
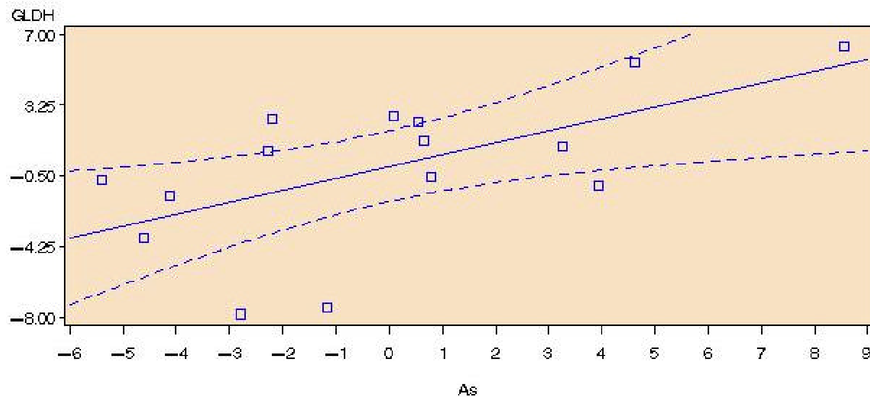
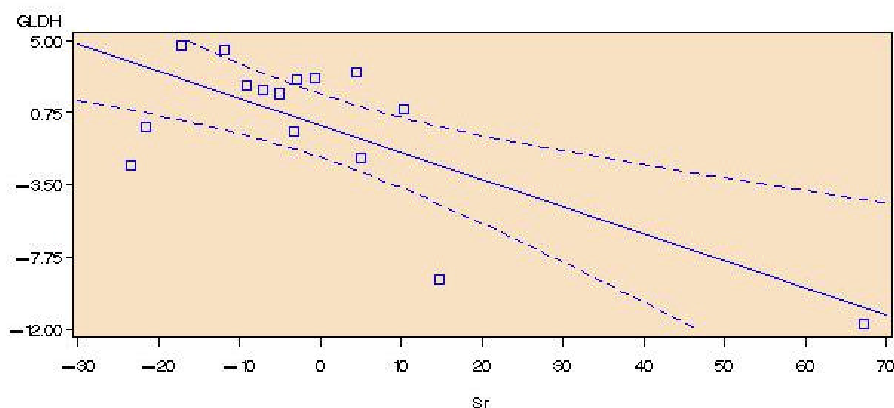


Figure 10.3 GS-DS-Female crabs. GLDH versus metals. Plots of individual metals, whereby, Cu explained 64%, As 7% and Sr 16% of the R^2 of 87%. Site equals (EQ) Gladstone (GS) and sex equals female (F) and condition equals diseased (DS).







10.3.5. All Gladstone female Non-diseased crabs

The regression analyses of the female non-diseased group also highlighted some strong relationships with some high R^2 values presented in table 10.5. No plots are presented for this group of crabs.

Table 10.5. Results of stepwise multiple regression of metals (independent) against immune responses (dependent) for all Gladstone female Non-diseased crabs. Results in *italics* indicate a two-point regression with a misleadingly high R^2 value. The direction of each metal in the final equation is positive, unless the name of the metal is underlined, indicating that the relationship was negative (i.e. immune response increased as the metal concentration decreased). 'Log' indicates that the variable was log(x+1) transformed.

Immune Response	R^2	p-value	Metals in final equation
ABR1	.462	.0053	-logAs
ABR2	.768	.0008	-logAg-logMn+Nd
mABR	.588	.0049	-logAs+Hg
Pheno		ns	
GLDH	.428	.0081	-Ba
logABR1	.690	.0009	-logAs-logCu
logABR2	.721	.0022	-logAs+Hg-Sr
logmABR	.793	.0004	-logAs+logHg-Sr
logPheno		ns	
logGLDH	*.863*	*.0003*	*-logFe-Ba+Nd+U*

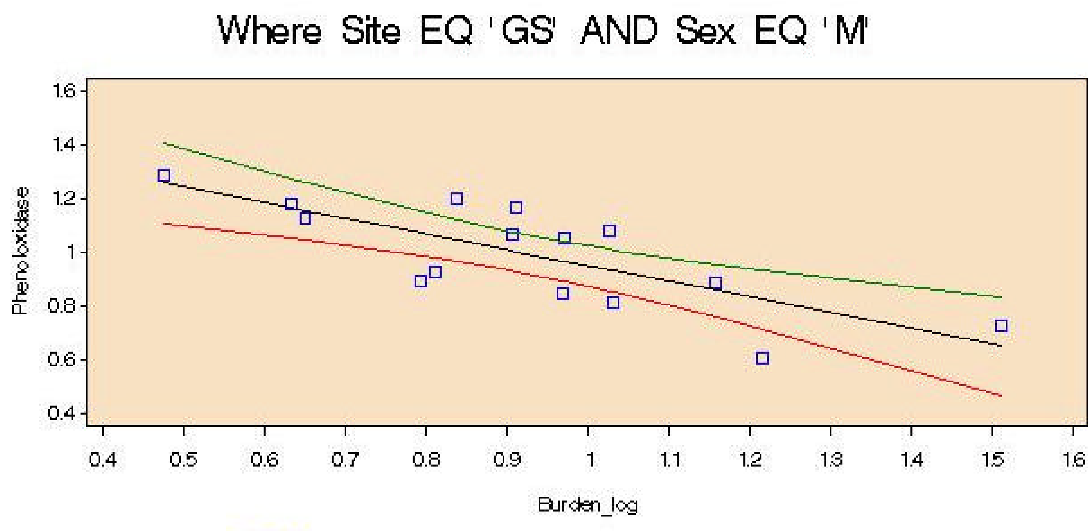
10.3.6. All Gladstone Male Non-diseased crabs

Like the female groups of Gladstone crabs regressions of metal levels and immune responses of the male group of non-diseased Gladstone crabs also identified a number of strong relationships as seen in Table 10.6. The plot for phenoloxidase is presented in Figure 10.4 as it represents a negative relationship with logBurden. i.e. immune response in this case increased as total metal burdens decreased.

Table 10.6. Results of stepwise multiple regressions of metals (independent) against immune responses (dependent) for all Gladstone Male Non-diseased crabs. Results in *italics* indicate a two-point regression with a misleadingly high R^2 value. Results in **bold** are illustrated in a multi-fit plot. The direction of each metal in the final equation is positive, unless the name of the metal is underlined, indicating that the relationship was negative (i.e. immune response increased as the metal concentration decreased). 'Log' indicates that the variable was log(x+1) transformed.

Immune Response	R^2	p-value	Metals in final equation
ABR1		ns	
ABR2	*.917*	*<.0001*	*logCo-logMn-logV+Mo*
mABR	.652	.0018	-logV+Mo
Pheno	**.588**	**.0008**	**-logBurden**
GLDH	*.887*	*<.0001*	*logAl+Fe-Ni*
logABR1		ns	
logABR2	*.890*	*<.0001*	*-logAl+logNi+logPb+V*
logmABR	.588	.0049	logCo-V
logPheno	.577	.0010	-logBurden
logGLDH	*.894*	*.0002*	*logAl+logZn+Cr+Fe*

Figure 10.4 Multifit plot of GS-ND-Male crabs. Phenoloxidase versus metals. Plots of individual metals, whereby, logBurden explained 59% of the R^2 of 59%. Site equals (EQ) Gladstone (GS) and sex equals male (M) (non diseased).



10.3.7. All Ayr Female crabs

Very few relationships were established in the Ayr female crabs between immune responses and metal levels. Relationships that exist had low R^2 values indicating that these relationships were not strong. Results are presented in Table 10.7 but no plots are demonstrated.

Table 10.7. Results of stepwise multiple regressions of metals (independent) against immune responses (dependent) for all Ayr Female crabs. The direction of each metal in the final equation is positive, unless the name of the metal is underlined, indicating that the relationship was negative (i.e. immune response increased as the metal concentration decreased). 'Log' indicates that the variable was log(x+1) transformed.

Immune Response	R^2	p-value	Metals in final equation
ABR1		ns	
ABR2		ns	
mABR		ns	
Pheno	.355	.0192	Mn
GLDH	.310	.0312	-Mo
logABR1		ns	
logABR2		ns	
logmABR		ns	
logPheno	.304	.0331	
logGLDH		ns	

10.3.8. All Ayr Male crabs

Results for the male non-diseased crabs (Table 10.8) are similar to those of the Ayr female crabs and indicate some weak relationships. Again no plots for individual metals are provided.

Table 10.8. Results of stepwise multiple regressions of metals (independent) against immune responses (dependent) for all Ayr Male crabs. The direction of each metal in the final equation is positive, unless the name of the metal is underlined, indicating that the relationship was negative (i.e. immune response increased as the metal concentration decreased). 'Log' indicates that the variable was log(x+1) transformed.

Immune Response	R^2	p-value	Metals in final equation
ABR1		ns	
ABR2		ns	
mABR		ns	
Pheno	.349	.0337	Mo
GLDH		ns	
logABR1		ns	
logABR2		ns	
logmABR		ns	
logPheno	.307	.0497	Mo
logGLDH	.546	.0147	logV

10.4 DISCUSSION

The results demonstrate that there is clearly a statistical relationship between metal levels and immune responses in Gladstone mud crabs and as expected, that very few relationships were established between metal levels and immune responses in the Ayr group of crabs. Therefore some metals and perhaps some other contaminants not present at Ayr are influencing the production of the two immune responses and the cellular enzyme in Gladstone crabs. Although in some cases relationships were not strong as can be seen by some of the r^2 values which are <0.5, there is an association between some immune responses and changes in some corresponding metal levels. On occasions (eg. phenoloxidase/logburden/GS-ND-M), this relationship is a negative

one i.e. as metal burden increases so phenoloxidase decreases. Smith and Johnston (1992) determined that exposure to polychlorinated biphenyls (PCBs) caused a decrease in PO levels in the common shrimp *Crangon crangon*. O'Halloran et al. (1998) commented that one part of the immune system can become quite active to compensate for chemical-induced immunosuppression of another part of the immune system. Zelikoff et al. (1995) also noted that it is also common to observe in fish, an enhanced immune response at low concentrations of heavy metals, followed by suppression at higher exposure levels. Unlike fish and other vertebrates, crustaceans and other invertebrates do not have a specific immune response (i.e. antibody or true immune memory) but rely on non-specific defences such as phagocytosis and antibacterial activity (Fries 1984). Therefore some care should be taken when making interclass comparisons of immune systems.

Results of metal analyses of Gladstone mud crabs suggest that these crabs have had a chronic exposure to a range of metals and metal concentrations. Low-level, long-term exposure to heavy metals appears to alter immunocompetence, exacerbating disease states by lowering resistance and allowing the invasion of infectious agents (Zelikoff and Cohen 1996). Most of the literature reporting the effect of contaminants on immune levels involves exposure of animals to acute high levels of these contaminants in a laboratory situation which, can have a completely different outcome to those results seen in naturally polluted systems. The complications of a synergistic or antagonistic effect of contaminants in the environment are difficult to reproduce in the laboratory.

Lowe and Fossato (2000) also used multi-stepwise regression analysis to explore causality between tissue contaminant burdens and lysosomal activity in the digestive cells of mussels (*Mytilus galloprovincialis*) from contaminated and reference sites. The results indicated that whilst activity of one immune enzyme correlated with body burdens of Hg, another enzyme in contrast correlated with a number of organic contaminants in combination with Fe or Zn. The results are similar to ours where positive correlations were established for a group of metals for one blood parameter yet often a completely different group of metals contributed to the total activity of one of the other enzymes. Lowe and Fossato (2000) also noted that where the immune responses generally gave good positive correlations with the organic contaminants they studied, the correlations with metals were more variable. That is, some metals initiated positive relationships (stimulatory) with immune response whereas others indicated a negative (inhibitory) response. They also recognised that contrasting immune responses to the same metal could be related to a synergism between the metal and other contaminants in polluted field sites, resulting in that metal becoming more toxic.

Metal speciation may also play a role in the effect of the metal on an immune response. Although most experimental studies examining immune parameters and metal exposure only use soluble metal forms, field animals are exposed to both soluble and particulate forms of a metal. Although Weiser (1968) (in Arumugam and Ravindranath 1987) suggested that food is the major source of copper which would be taken in as particulate matter in decapod crustaceans, other authors suggest that the gills are the main sites of uptake of soluble copper (Truchot and Rtal 1998, Soegianto et al. 1999). Therefore the route of uptake and the pathway of detoxification taken by a metal may have different effects on immune responses.

In conclusion our results indicate that there is a significant relationship between some immune responses/cellular enzymes and tissue metal levels in the Gladstone groups of crabs. Although we cannot determine direct cause and effect, the regressions indicate that there is a chemical/physical process whereby immune responses change as a function of a change in the concentration of one or more metals and perhaps other, yet undetermined contaminants.

Conclusion

Results of stepwise multiple regression analyses suggest that in the Gladstone crabs there are many statistical relationships between metal levels and immune responses/cellular enzymes. There were fewer, weaker relationships identified in the Ayr crabs in comparison to the Gladstone groups, although at times established relationships were inhibitory rather than stimulatory (i.e. indicating exposure to a metal causing inhibition rather than production of an immune factor). Although the regressions do not prove cause and effect they do provide proof of relationships whereby blood parameters can change in response to a change in metal accumulations in Gladstone crab tissues.

CHAPTER 11. Sources of Contaminants

11.1 INTRODUCTION

The metal analyses of mud crabs, has emphasized elevated metal levels in particular copper and zinc, in Gladstone mud crabs compared to those from other areas. Prange (1999) also recorded elevated copper levels in a variety of seagrass species in Port Curtis samples in comparison to those in Moreton Bay. It became apparent that the overwhelming question was “What is the source of the elevated copper/metal loads”? There are three major routes of uptake of metals into aquatic organisms. The first is via the gills from metals in the water column. The second route is via ingestion of metals bound to sediments and the third route is the food source. Sediment analyses of Port Curtis and Ayr have shown few differences exist in metal levels from the two locations and that levels are below recommended ANZECC (1998) guidelines (Andersen and Melzer, unpub.). Water quality in Port Curtis has determined only occasionally elevated levels of some metals (Sinclair, Knight & Merz, 1999). As mud crabs spend part of their life cycle in permanent burrows, however, we questioned if there might exist a separate microcosm within these burrows, which may be a source for the elevated metals. The burrows are thought to be permanent structures used by successive generations of mud crabs and probably protect the crabs during moulting and mating periods, when they are more vulnerable (Fielder and Heasman, 1978).

The use of stable isotopes as an alternative to gut content analyses has been used successfully to elucidate aquatic food web interactions (Fantle et al. 1999, Kang et al 1999). The elements C, N, S, H and O all have more than one isotope, and the isotopic compositions of natural materials (eg. mud crab muscle tissue) can be measured with great precision with a mass spectrometer (Petersen and Fry, 1987). Isotopic compositions change in predictable ways as these elements cycle upward through the food web (Petersen and Fry, 1987). More recently, however, isotopes offer the potential not only to quantify trophic position, but allow the movement of contaminants to be traced through food webs (Schindler et al. 1995). These predicted isotopic changes, can then be exploited to establish what organisms constitute the mud crab diet and what if any correlations exist between metal concentrations in these organisms and the mud crab. Analyses of mud crab tissue samples from both Ayr and Gladstone for stable isotopes of carbon and nitrogen would establish if any differences exist in the food webs from the two locations, which might also explain the contrasting copper tissue levels. Isotopic compositions are expressed as Delta (?) values, which are parts per thousand differences from a standard (?X = (R_{sample}/ $R_{standard}$ - 1) x 1000 (‰)) (Petersen and Fry, 1987).

11.2 METHODS

11.2.1 Burrow sampling

Three permanent mud crab burrows from three different sites within Port Curtis (Sites 1, 2, and 3) were selected for sampling. Burrows were located in the high intertidal zone with the majority being inundated with water only on the high spring tides, although they extend below the low tide water table and always contain some water. Mud crabs were occasionally resident within the burrows sampled, however, no crabs

were harmed during sampling. Equipment used for collection was acid washed and rinsed in distilled water or soaked overnight in seawater prior to use to remove contaminants. Corrugated flexible tubing inserted into a rubber ball was fed into the burrow to act as a guide for the introduction of sampling equipment. The depth of the burrows, temperature and dissolved O_2 of the water in the burrows, were measured by inserting probes from a hand held TPS 90 FLMV water quality monitoring unit. A water sample from the bottom of the burrow was then collected using tubing connected to a hand operated pump and placed into acid washed containers. Samples were transported on ice to the lab where the PH and salinity were measured prior to filtering. Nitric acid (70% m/V) was added to each sample, which was kept on ice until screened for a select number of metals via ICP-MS in semi-quant mode.

Duplicate core sediment samples (approximately 5cm depth) were also taken from the bottom of each burrow and triplicate samples from adjacent mud flats at Site 1, using a length of conduit. Samples were placed in zip lock bags and placed on ice for transport prior to freezing. Samples were dried at 105ºC, manually ground and sieved (0.5mm) and analysed according to Method USEPA 3051: Microwave Assisted Acid Digestion of Sediments, Sludges, Soils and Oils. A representative sample of up to 1g was digested in 10mL of concentrated nitric acid for 10 minutes using microwave heating with a suitable laboratory microwave unit. After cooling, the vessel contents were diluted to volume and analysed by the appropriate method (eg. ICP-AES /ICP-MS).

11.2.2 Isotope sampling

Mud crab muscle tissue from five non-diseased male crabs from both Ayr and Gladstone was collected as previously described in Chapter 7. Samples were stored frozen prior to being oven dried @ 70ºC for 72hours and ground with a mortar and pestle. At a NATA certified laboratory (CSIRO NSW) samples were oxidised at a high temperature and the resultant CO_2 and N_2 analysed with a continuous flow-isotope ratio mass spectrometer (Europa Tracermass and Roboprep, Crewe, U.K.). Ratios of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ to be expressed as the relative per ml (‰) difference between the sample and conventional standards (PDB carbonate and N_2 in air) where:

$$?X = (R_{sample}/ R_{standard} - 1) \times 1000\ (‰)$$

where $X = {}^{13}C$ or ${}^{15}N$ and $R = {}^{13}C/{}^{12}C$ or ${}^{15}N/{}^{14}N$.

11.3 RESULTS

11.3.1 Burrow sampling

The results of the metal analyses of the water samples are depicted in Table 11.1 and the sediment samples in Table 11.2. The levels of all metals were fairly low in both water and sediments being below ANZECC (1992) and (1998) guidelines respectively, in all cases.

Table 11.1 Metal levels of water samples from mud crab burrows from Port Curtis.

Data in ug/L										
Sites/burrow	**Cu**	**Al**	**P**	**Cr**	**Mn**	**Fe**	**Co**	**Ni**	**Zn**	**Cd**
Site 1/ Burrow 1	<1	<1	29	<1	169	68	<1	2	<1	<1
Site 1/ Burrow 2	1	<1	24	<1	60	37	1	2	<1	<1
Site 1/ Burrow 3	<1	<1	42	1	105	408	1	2	<1	<1
Site 2/ Burrow 1	<1	2	40	<1	69	1370	3	3	<1	<1
Site 2/ Burrow 2	<1	1	42	<1	67	72	1	3	<1	<1
Site 2/ Burrow 3	<1	<1	36	<1	600	38	1	3	<1	<1
Site 3/ Burrow 1	1	<1	48	<1	102	202	1	2	<1	<1
Site 3/ Burrow 2	<1	1	25	<1	23	2	<1	1	<1	<1
Site 3/ Burrow 3	1	1	26	1	30	5	2	3	<1	<1
ANZECC 1992	5	NR	NR	50	NR	NR	NR	15	50	2

Table 11.2 Metal levels of sediment core samples from burrows and adjacent mud flats at Site 1.

Sample	**Cu**	**Zn**
Results in dry wgt	**(µg/g)**	**(µg/g)**
mudflat 1	14	31
mudflat 2	14	31
mudflat 3	13	31
burrow 1 a	10	19
burrow 1 b	13	23
burrow 2 a	16	28
burrow 2 b	15	35
burrow 3 a	13	20
burrow 3 b	18	29
ANZECC 1998	65	200
screening guideline		

The physiochemical properties of water in the burrows, is described in Table 11.3. The water in all the burrows was sulphidic in nature and acidic (in comparison to ocean water pH = 8.2), with an extremely, low dissolved oxygen (DO_2). As expected, a positive correlation was established between the pH and the DO_2 in the bottom water (DO_2 bottom = -30.09 + 4.5116 * PH. Correlation : r = 0.74510).

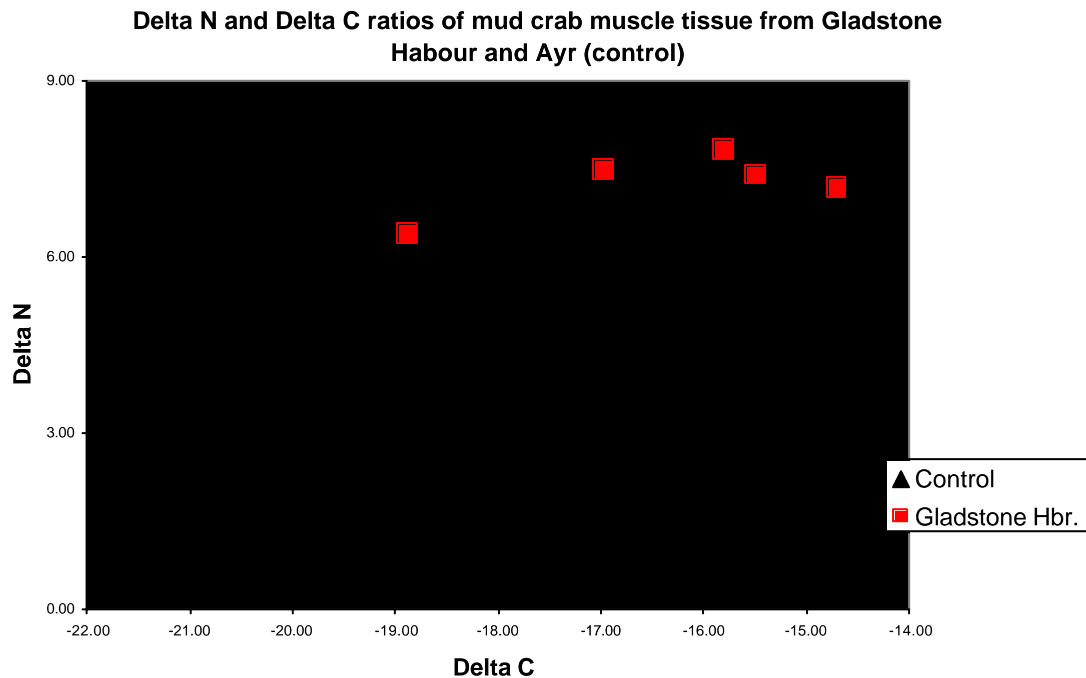
Table 11.3 Physiochemical properties of water in mud crab burrows in Port Curtis.

Site	**pH**	**Sal**	**DO_2mg/L Bottom**	**DO_2mg/L Top**	**Depth of burrow mm**	**Temp C**
Site 1/ Burrow 1	7	36.7	1	1.3	920	NR
Site 1/ Burrow 2	6.98	33.6	1	2.9	630	18.2
Site 1/ Burrow 3	6.88	39.8	0.4	1.2	980	19
Site 2/ Burrow 1	6.87	37	1.5	1.6	700	18
Site 2/ Burrow 2	6.74	40.8	0.8	1	500	19
Site 2/ Burrow 3	7.15	40.9	1.1	2.1	1400	18.2
Site 3/ Burrow 1	6.94	33.7	1.6	2.4	1570	21.9
Site 3/ Burrow 2	7.29	38.7	4.1	4.8	1740	20.1
Site 3/ Burrow 3	7.24	37	2.3	5	700	21.5

11.3.2 *Isotope sampling*

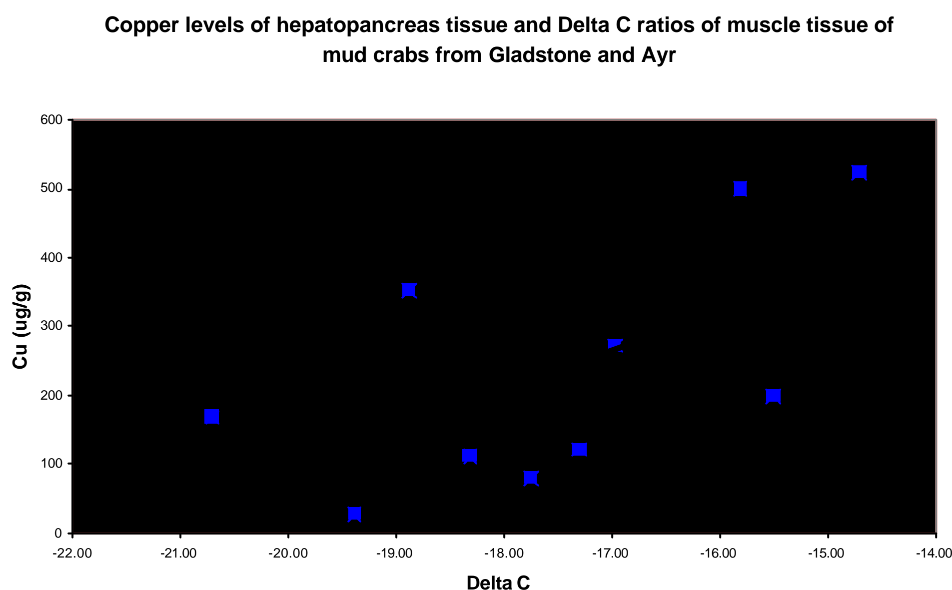
The Delta N and Delta C results for mud crab tissue from both Gladstone and Ayr are plotted in Figure 11.1. The Delta N readings for both groups of crabs are fairly similar indicating that both groups of crabs have a similar trophic position i.e. they are second level feeders consuming another animal, which consumes plant material. The Delta C readings show that apart from one outlier, there is a separation between the two groups. This tends to indicate that the source of carbon for the two groups may be different and suggests that the Gladstone crabs could be consuming something, which is not in the diet of the Ayr crabs. Sample numbers in this pilot study were, however, low and repeated sampling would be required to determine a clear separation.

Figure 11.1 Delta C and Delta N ratios of mud crab muscle tissue from Ayr (control) and Gladstone.



The results of the Delta C readings were then correlated with the copper levels in the tissues of the same crabs. A positive correlation was established between Delta C ratios and copper levels in hepatopancreas ($r = 0.605$, Figure 11.4). A weaker correlation was also established between Delta C levels and muscle copper levels ($r = 0.534$). The results although preliminary, suggest that the Gladstone crabs may be consuming something in their diet which is enriched with copper, but which is not found in the diet of Ayr crabs.

Figure 11.2 Hepatopancreas copper levels and Delta C ratios of mud crab muscle tissue from Ayr and Gladstone (r = 0.605).



11.4 DISCUSSSION

The burrow of the mud crab is an interesting habitat. Some burrows were over 1m in depth and often consisted of a number of chambers. The normal dissolved oxygen and PH for saline ocean water is around 6mg/L and 8.2 respectively, as recommended by ANZECC (1992) guidelines. Interestingly the dissolved oxygen in the burrows fell well below this level particularly in the acidic conditions. Burrows are often used as a safe haven during a moult, one of the most physiologically stressful periods in a crustacean life cycle. Davenport and Wong (1987) from invitro experiments suggested that the mud crab is ill adapted to low pO_2. Therefore considering that O_2 demand during this time must be at a premium, it is surprising that the mud crab has selected and adapted to such unfavourable conditions. The finding of very low concentrations of metals in sediments and water in mud crab burrows, suggests that the two media are not the source of elevated metal concentrations in the Gladstone mud crabs.

The source of elevated copper in Gladstone mud crabs if not water and sediments could be the food web. Chou et al. 2000 found no relationships between digestive gland metals and associated sediments in the American lobsters (*Homarus americanus*) they studied. They proposed that the major intake of metals in lobsters is via the diet and involved bioaccumulation along the food chain. The lower Delta C diet (Gladstone) has the higher copper content, which could be due to the presence of a particular dietary source (bivalve, snail etc.) absent in the high Delta C diet (Ayr). Although the mud crab is known to feed on slow moving benthic invertebrates their exact food web is not fully defined. Bivalves, gastropods (snails) and other crustaceans are considered to make up a large part of the mud crab diet (Hill 1976). Although we have suggested in this preliminary investigation a probable relationship between the copper concentrations in mud crabs and potentially a part of their diet, more research is required to isolate which food sources could be involved. If the diet

is the source of elevated metals in Gladstone mud crabs then the source of accumulation of copper in these plants or animals, however, poses further questions.

Conclusion

The results of metal analyses of water and sediments from the permanent burrows of Gladstone mud crabs, indicates low levels of metals exist in the burrows and therefore burrows (i.e. water and sediments) are unlikely to be a source of elevated metals in Gladstone mud crabs. Stable isotopes of carbon and nitrogen were used as an alternative to gut content analyses to determine if differences existed in the diets of Gladstone crabs compared to Ayr crabs which, might explain the contrasting tissue metal results. Although the results are preliminary, they suggest that the Gladstone crabs may be consuming something in their diet, which is enriched in copper but is not available in the Ayr mud crab diet. Further research is required in this area.

Chapter 12. Management

Objective: *Depending on the cause/s isolated/identified, work out a management strategy to lessen the effects of rust spot shell disease.*

When the shell disease investigation first began, the probability that the disease was caused by an infectious organism was foremost on the list of differential diagnoses. The possibility of a new contagious disease in mud crabs similar to that of EUS (epizootic ulcerative syndrome) in fish or white spot in prawns could have far reaching implications. Unsightly gross shell lesions could have had a devastating effect on the mud crab industry. If this disease had been able to cross infect other crustaceans, the lucrative prawn aquaculture industry would also have been threatened. The possibility that the disease had been introduced in ballast water or on hull fouling in Port Curtis, an international trades port, could have implications for other ports in other countries with further spread via these same routes.

Our research indicates, however, that the rust spot syndrome does not have an infectious cause and may be in fact environmental. This brings to the fore a new set of factors which must be considered in the management of the disease. The elevated prevalence of shell disease appears to be restricted to Port Curtis and Fitzroy River, which also have generally elevated metal burdens (in particular copper and zinc) in their mud crab populations. A number of associations have been established which indicate that elevated metals levels could be implicated in the cause of rust spot shell lesions. Fortunately the metal levels are below those levels recommended for metals in ANZFSC (2000) food standards code and therefore the crab muscle meat is considered suitable for consumption. Levels of some metals are, however, above proposed Generally Expected Levels (GEL's) put forward by ANZFA as a guideline to the food standards code. Therefore metal levels should continue to be monitored. In a small number of cultures the whole crab including the hepatopancreas is also consumed and in these cases it is difficult to apply the present food standards code, which only applies to mud crab meat. However, as mud crabs are considered to be only an occasionally consumed food health risks in terms of metal concentrations from the consumption of whole crab are likely to be minimal. The fact that less than 1% of mud crabs marketed have perforated lesions means there should be little effect on marketability from crabs with unsightly gross shell lesions and a change in marketing strategy is not required.

There is, however a question over the comparative "health" of Gladstone crabs compared to those from Ayr and hence the quality of the ecological environment of Port Curtis. The stimulated immune/cellular enzyme levels and the findings of statistical relationships between those immune levels and metal concentrations suggest a higher level of "stress" in Gladstone mud crabs. The possible stress factor was highlighted in the Gladstone diseased group of crabs in particular with high within group variation in metal concentrations, suggesting an inability to regulate metal levels. It is possible that the stimulated immune and cellular enzyme responses are a normal reaction to the mud crabs environmental conditions and a normal means of coping with a contaminant challenge. The effect of elevated metal and immune levels on reproduction, growth or general wellbeing of the mud crab has not been considered in the scope of this project, but perhaps should also be investigated.

There is also the question of why the syndrome appeared in 1994. Apart from a lower rainfall around this period, no major changes occurred in Port Curtis at this time. During the period from 1990-2000, however, the shipping tonnage doubled. We might also assume that industry production, population, urbanisation and human activity increased steadily over this time, and hence also contaminant inputs into Port Curtis. It would not appear unreasonable to suggest that the stressed/abnormal state of Gladstone mud crabs is related in part at least to their heavy metal burdens and that these are in turn related to an increase in human activity around Port Curtis.

It is apparent that there is an urgent need to

1. Establish if elevated metal levels exist in other biota in Port Curtis.
2. Determine the source of elevated metal levels.

The Centre for Environmental Management is pursuing research into metal levels in other biota in Port Curtis, as well as using stable isotopes to establish the food web of the mud crab, in order to determine the source of elevated metals. Preliminary results of metal levels in fiddler crabs (*Uca coarctata*) from a number of sites in Port Curtis in comparison to reference sites suggest elevated copper concentrations in fiddler crabs from inner harbour sites (Andersen and Melzer unpub.). Prange (1999) also recorded elevated copper levels in a variety of seagrass species in Port Curtis samples in comparison to Moreton Bay. There is therefore also an urgent need to go beyond the food sources of the mud crab to the origin of contamination of those food sources eg. urban, industrial or agricultural.

A conservative management approach would also be to

3. Reduce the flow of metals into Port Curtis from all sources by reviewing the current point and area sources of discharge.
4. Continue to monitor crab health in terms of metal burdens immune responses and the incidence rust spot shell lesions.

Further research into the ecosystem health of Port Curtis will determine what management strategies are required to address this issue.

Benefits

The investigation into a new shell disease in Port Curtis mud crabs was necessary to determine the extent of the disease and the risk it imposed to the commercial and recreational fishing sectors as well as other crustaceans and the surrounding ecosystem. The outcome that the disease appears to be a non-infectious one has direct benefits to the local community and fishing sector as well as the aquaculture industry. Containment of a disease outbreak is no longer an issue. That there are unlikely to be any potential health risks from eating mud crab muscle meat in terms of metal levels and that presentation is unaffected is advantageous to all consumers of Port Curtis mud crabs, both commercially and recreationally.

The discovery of elevated metal levels in Port Curtis biota has prompted questions to be raised about the state of ecosystem health in the harbour. Previous harbour monitoring focused mainly on water and sediment quality, which often only gives a snapshot in time in terms of the state of the environment. Contaminant analyses of biota in the harbour can give a better picture of bioaccumulation and possible contaminant pathways which physiochemical analyses fails to deliver. A number of projects involving monitoring contaminants in Port Curtis biota have begun or are proposed, due to the findings of the mud crab project highlighting possible environmental issues in the harbour. The findings of these projects will benefit the community (industry, environment and population) as a whole.

Some of the data gathered in this study has given us an insight into the biology of the mud crab as a species in terms of pathology and immune levels which, may be used in future research and aquaculture of this species.

Further development

The Centre for Environmental Management has begun two small projects as a result of the findings of this study. The first project titled “Fiddler crabs as potential Bioindicators in Port Curtis” involves metal analyses of fiddler crabs and sediments from 13 sites around Port Curtis to determine if there are site differences in metal levels. The second project “An investigation into the source of elevated metal levels in Port Curtis mud crabs in association with a food web analyses” involves establishing the food web of the mud crab using stable isotopes and then analysing the biota in that food web for metal levels. More funding is required, however, to further investigate this area. Industry sponsorship has also been gained to support a Masters student associated with the findings of the project.

This project has highlighted a need for further research into the migration and movement habits of mud crabs of which little data exist at present. An annual sampling for the prevalence of shell disease is also recommended to record any future changes in the prevalence of rust spot shell lesions.

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APPENDICES

Appendix 1. Intellectual Property
There is no intellectual property arising from this project. All information generated is in the public domain.

Appendix 2. Staff
Staff employed on the project were:

Centre for Environmental Management, Central Queensland University:
Leonie Andersen (Research Officer, Principal Investigator)
Jill Campbell (Research Assistant)
Karen Boundy (Research Assistant)
Andrew Davis (Coxswain)
Lee Hackney (Centre Manager)

Oonoonba Veterinary Laboratory, Queensland Department of Primary Industry:
Dr. John Norton Veterinary Pathologist, Investigator)
Naomi Levy (Technician)

Appendix 3.
A reprint of the article "A new shell disease in the mud crab *Scylla serrata* from Port Curtis, Queensland (Australia)" (2000) by Andersen L.E., Norton J.H. and Levy N.H. *Diseases of Aquatic Organisms* Vol. 43: 233-239.

Appendix 4.

Elements, atomic number and symbol as listed in the periodic table.
Atomic
Number- Name- Symbol

105	??	Ha
104	??	Rf
89	Actinium	Ac
13	Aluminum	Al
95	Americium	Am
51	Antimony	Sb
18	Argon	Ar
33	Arsenic	As
85	Astatine	At
56	Barium	Ba
97	Berkelium	Bk
4	Beryllium	Be
83	Bismuth	Bi
5	Boron	B
35	Bromine	Br
48	Cadmium	Cd
20	Calcium	Ca
98	Californium	Cf
6	Carbon	C
58	Cerium	Ce
55	Cesium	Cs
17	Chlorine	Cl
24	Chromium	Cr
27	Cobalt	Co
29	Copper	Cu
96	Curium	Cm
66	Dysprosium	Dy
99	Einsteinium	Es
68	Erbium	Er
63	Europium	Eu
100	Fermium	Fm
9	Fluorine	F
87	Francium	Fr
64	Gadolinium	Gd
31	Gallium	Ga
32	Germanium	Ge
79	Gold	Au
72	Hafnium	Hf
2	Helium	He
67	Holmium	Ho
1	Hydrogen	H
49	Indium	In
53	Iodine	I
77	Iridium	Ir
26	Iron	Fe
36	Krypton	Kr
57	Lanthanum	La
103	Lawrencium	Lr
82	Lead	Pb
3	Lithium	Li
71	Lutetium	Lu
12	Magnesium	Mg
25	Manganese	Mn
101	Mendelevium	Md
80	Mercury	Hg
42	Molybdenum	Mo
60	Neodymium	Nd
10	Neon	Ne
93	Neptunium	Np
28	Nickel	Ni
41	Niobium	Nb
7	Nitrogen	N
102	Nobelium	No
76	Osmium	Os
8	Oxygen	O
46	Palladium	Pd
15	Phosphorus	P
78	Platinum	Pt
94	Plutonium	Pu
84	Polonium	Po
19	Potassium	K
59	Praseodymium	Pr
61	Promethium	Pm
91	Protactinium	Pa
88	Radium	Ra
86	Radon	Rn
75	Rhenium	Re
45	Rhodium	Rh
37	Rubidium	Rb
44	Ruthenium	Ru
62	Samarium	Sm
21	Scandium	Sc
34	Selenium	Se
14	Silicon	Si
47	Silver	Ag
11	Sodium	Na
38	Strontium	Sr
16	Sulfur	S
73	Tantalum	Ta
43	Technetium	Tc
52	Tellurium	Te
65	Terbium	Tb
81	Thallium	Tl
90	Thorium	Th
69	Thulium	Tm
50	Tin	Sn
22	Titanium	Ti
92	Uranium	U
23	Vanadium	V
74	Wolfram	W
54	Xenon	Xe
70	Ytterbium	Yb
39	Yttrium	Y
30	Zinc	Zn
40	Zirconium	Zr

Appendix 5.

Mean (+ 1 SE) concentration of each metal in yr2000 hepatopancreas samples for crabs from each location (CT/GS), sex (M/F) and condition (DS/ND) in mg/kg wet wgt.

Site/sex/ condition	No	Ag	Al	As	Ba	Cd	Ce	Co	Cr	Cu
CT-F-ND	15	0.81 (0.16)	2.61 (0.24)	19.10 (2.23)	0.16 (0.02)	1.02 (0.21)	0.07 (0.01)	0.60 (0.06)	0.28 (0.03)	69.50 (14.31)
CT-M-ND	15	1.07 (0.11)	1.97 (0.22)	11.27 (1.62)	0.40 (0.10)	0.70 (0.10)	0.15 (0.03)	0.68 (0.10)	0.15 (0.02)	122.43 (16.50)
GS-F-DS	15	1.75 (0.49)	4.45 (1.97)	6.17 (1.00)	0.43 (0.14)	0.96 (0.37)	0.65 (0.18)	1.61 (0.57)	0.85 (0.26)	247.68 (56.69)
GS-F-ND	15	1.06 (0.10)	1.91 (0.39)	7.67 (0.42)	0.18 (0.02)	0.51 (0.10)	0.13 (0.03)	0.80 (0.12)	0.48 (0.03)	293.37 (38.61)
GS-M-ND	15	0.68 (0.13)	2.66 (0.55)	8.36 (0.67)	0.25 (0.03)	0.38 (0.04)	0.28 (0.09)	0.97 (0.09)	0.32 (0.04)	392.98 (43.67)

Site/sex/ condition	No	Fe	Ga	Gd	Hg	La	Mn	Mo	Nd	Ni	Pb
CT-F-ND	15	119.63 (24.78)	0.05 (0.00)	0.05 (0.00)	0.17 (0.02)	0.06 (0.01)	8.90 (0.97)	0.91 (0.08)	0.05 (0.00)	1.28 (0.25)	0.05 (0.00)
CT-M-ND	15	78.65 (9.39)	0.05 (0.00)	0.05 (0.00)	0.07 (0.01)	0.12 (0.03)	4.43 (0.83)	0.53 (0.08)	0.06 (0.01)	0.91 (0.20)	0.06 (0.01)
GS-F-DS	15	49.77 (11.41)	0.06 (0.01)	0.06 (0.01)	0.12 (0.04)	0.46 (0.13)	10.60 (4.58)	1.49 (0.64)	0.26 (0.07)	1.92 (0.75)	0.09 (0.03)
GS-F-ND	15	73.19 (12.97)	0.05 (0.00)	0.05 (0.00)	0.07 (0.01)	0.10 (0.02)	5.57 (0.72)	0.71 (0.13)	0.06 (0.01)	0.88 (0.11)	0.05 (0.00)
GS-M-ND	15	64.30 (9.71)	0.05 (0.00)	0.05 (0.00)	0.07 (0.01)	0.19 (0.06)	4.15 (0.44)	0.57 (0.06)	0.11 (0.04)	0.98 (0.21)	0.06 (0.01)

Site/sex/ condition	No	Pr	Rb	Sb	Sm	Sn	Sr	Te	U	V	Zn
CT-F-ND	15	0.05 (0.00)	0.05 (0.00)	0.05 (0.00)	0.05 (0.00)	0.05 (0.00)	58.30 (8.46)	0.05 (0.00)	0.23 (0.11)	0.38 (0.06)	30.56 (3.45)
CT-M-ND	15	0.05 (0.00)	0.05 (0.00)	0.05 (0.00)	0.05 (0.00)	0.05 (0.00)	47.53 (12.99)	0.06 (0.01)	0.13 (0.04)	0.27 (0.03)	44.16 (3.87)
GS-F-DS	15	0.08 (0.02)	0.05 (0.00)	0.06 (0.01)	0.06 (0.01)	0.12 (0.04)	27.58 (5.71)	0.06 (0.01)	0.48 (0.25)	0.78 (0.30)	44.31 (7.58)
GS-F-ND	15	0.05 (0.00)	0.05 (0.00)	0.05 (0.00)	0.05 (0.00)	0.05 (0.00)	39.16 (8.21)	0.05 (0.00)	0.17 (0.08)	0.36 (0.02)	71.72 (6.51)
GS-M-ND	15	0.06 (0.01)	0.05 (0.00)	0.05 (0.00)	0.05 (0.00)	0.05 (0.00)	25.16 (5.11)	0.05 (0.00)	0.37 (0.19)	0.45 (0.02)	46.56 (3.05)

Appendix 6.
Mean concentration (mg/kg wet wt) of metals in hepatopancreas of mud crabs from Ayr and Gladstone in 1999 and 2000.

	Ayr 1999	Ayr 2000	Gladstone 1999	Gladstone 2000
	(10)	(30)	(20)	(45)
As	11.740	15.187	8.075	7.398
Cd	0.529	0.859	0.366	0.615
Ce	0.097	0.109	0.092	0.351
Co	0.402	0.637	1.112	1.126
Cr	0.263	0.215	0.151	0.551
Cu	82.960	95.963	197.900	311.342
Fe	80.000	99.137	94.960	62.420
Hg	0.065	0.122	0.054	0.085
La	0.069	0.089	0.065	0.250
Mn	2.230	6.663	13.235	6.773
Nd	0.050	0.056	0.050	0.146
Pb	0.302	0.055	0.156	0.068
Pr	0.050	0.050	0.050	0.064
Rb	0.742	0.050	0.641	0.050
Sb	0.050	0.050	0.050	0.053
S	0.050	0.050	0.050	0.053
Sn	0.058	0.050	0.085	0.076
Sr	8.290	52.913	29.080	30.633
U	0.149	0.182	0.314	0.342
V	0.157	0.327	0.254	0.529
Zn	28.530	37.360	63.855	54.198

Appendix 7.
Mean (+ 1 SE) concentration of each metal in yr2000 hepatopancreas samples for female crabs from each location (CT, Control; GS, Gladstone; FZ, Fitzroy) and condition (DS/ND) in mg/kg wet wgt.

Site/Condition	No.	Ag	Al	As	Ba	Cd	Ce	Co	Cr	Cu	Fe
CT-ND	15	0.81 (0.16)	2.61 (0.24)	19.1 (2.23)	0.16 (0.02)	1.02 (0.21)	0.07 (0.01)	0.6 (0.06)	0.28 (0.03)	69.5 (14.31)	119.63 (24.78)
FZ-ND	9	2.34 (0.21)	1.24 (0.17)	10.77 (0.69)	0.46 (0.07)	1.07 (0.33)	0.18 (0.02)	1.02 (0.16)	0.23 (0.02)	263.56 (22.99)	50.68 (9.76)
GS-DS	15	1.75 (0.49)	4.45 (1.97)	6.17 (1.00)	0.43 (0.14)	0.96 (0.37)	0.65 (0.18)	1.61 (0.57)	0.85 (0.26)	247.68 (56.69)	49.77 (11.41)
GS-ND	15	1.06 (0.10)	1.91 (0.39)	7.67 (0.42)	0.18 (0.02)	0.51 (0.10)	0.13 (0.03)	0.8 (0.12)	0.48 (0.03)	293.37 (38.61)	73.19 (12.97)

		Ga	Gd	Hg	La	Mn	Mo	Nd	Ni	Pb	Pr
CT-ND	15	0.05 (0.00)	0.05 (0.00)	0.17 (0.02)	0.06 (0.01)	8.9 (0.97)	0.91 (0.08)	0.05 (0.00)	1.28 (0.25)	0.05 (0.00)	0.05 (0.00)
FZ-ND	9	0.05 (0.00)	0.05 (0.00)	0.09 (0.01)	0.14 (0.02)	4.07 (0.67)	0.62 (0.08)	0.06 (0.01)	3.01 (0.56)	0.05 (0.00)	0.05 (0.00)
GS-DS	15	0.06 (0.01)	0.06 (0.01)	0.12 (0.04)	0.46 (0.13)	10.6 (4.58)	1.49 (0.64)	0.26 (0.07)	1.92 (0.75)	0.09 (0.03)	0.08 (0.02)
GS-ND	15	0.05 (0.00)	0.05 (0.00)	0.07 (0.01)	0.1 (0.02)	5.57 (0.72)	0.71 (0.13)	0.06 (0.01)	0.88 (0.11)	0.05 (0.00)	0.05 (0.00)

		Rb	Sb	Sm	Sn	Sr	Te	U	V	Zn
CT-ND	15	0.05 (0.00)	0.05 (0.00)	0.05 (0.00)	0.05 (0.00)	58.3 (8.46)	0.05 (0.00)	0.23 (0.11)	0.38 (0.06)	30.56 (3.45)
FZ-ND	9	0.05 (0.00)	0.05 (0.00)	0.05 (0.00)	0.05 (0.00)	38.68 (9.09)	0.05 (0.00)	0.19 (0.04)	0.25 (0.01)	57.99 (5.11)
GS-DS	15	0.05 (0.00)	0.06 (0.01)	0.06 (0.01)	0.12 (0.04)	27.58 (5.71)	0.06 (0.01)	0.48 (0.25)	0.78 (0.30)	44.31 (7.58)
GS-ND	15	0.05 (0.00)	0.05 (0.00)	0.05 (0.00)	0.05 (0.00)	39.16 (8.21)	0.05 (0.00)	0.17 (0.08)	0.36 (0.02)	71.72 (6.51)

Legend for Appendixes

CT = Control
GS = Gladstone
DS = Diseased
ND = Non-diseased

Appendix 8.
Mean (+ 1 SE) concentration of each metal in yr2000 muscle samples for crabs from each location (CT/GS), sex (M/F) and condition (DS/ND) in mg/kg wet wgt.

Level	n	Al	As	Ba	Cr	Cu	Fe
CT-F-ND	10	0.400 + 0.055	16.210 + 1.721	0.121 + 0.032	0.232 + 0.023	12.480 + 1.877	4.910 + 0.883
CT-M-ND	10	1.107 + 0.187	7.570 + 1.005	0.252 + 0.126	0.298 + 0.072	4.270 + 0.256	5.530 + 0.983
GS-F-DS	10	3.390 + 0.654	7.700 + 0.912	0.102 + 0.030	0.238 + 0.022	12.220 + 1.622	6.380 + 1.506
GS-F-ND	10	6.540 + 1.227	9.600 + 0.649	0.061 + 0.011	0.257 + 0.017	12.010 + 1.615	8.400 + 1.196
GS-M-ND	10	12.580 + 2.971	7.230 + 0.987	0.108 + 0.027	0.288 + 0.028	13.150 + 2.576	13.670 + 3.385

Level	n	Hg	Mn	Sr	V	Zn
CT-F-ND	10	0.111 + 0.015	4.930 + 1.093	11.300 + 2.748	0.050 + 0.000	87.220 + 2.770
CT-M-ND	10	0.056 + 0.006	1.861 + 0.743	7.600 + 2.188	0.050 + 0.000	61.060 + 2.690
GS-F-DS	10	0.050 + 0.000	4.232 + 2.132	10.690 + 2.604	0.050 + 0.000	80.050 + 3.240
GS-F-ND	10	0.067 + 0.012	2.625 + 1.058	4.940 + 0.871	0.050 + 0.000	87.800 + 2.586
GS-M-ND	10	0.050 + 0.000	3.188 + 0.662	10.677 + 1.577	0.088 + 0.014	75.050 + 4.710

Appendix 9.
Mean (+ 1 SE) concentration of Cu, Zn and Al in yr1999 and yr2000 muscle samples for crabs from each location (CT/GS), sex (M/F) and condition (DS/ND) in mg/kg wet wgt.

Year	Site	Sex	Condition	n	Cu	Zn	Al
1999	CT	M	ND	10	3.760 + 0.206	57.280 + 1.103	1.133 + 0.386
2000	CT	M	ND	10	4.270 + 0.256	61.060 + 2.690	1.107 + 0.187
1999	GS	M	ND	4	9.900 + 1.792	53.400 + 2.354	1.815 + 0.420
2000	GS	M	ND	10	13.150 + 2.576	75.050 + 4.710	12.580 + 2.971
1999	GS	F	ND	6	7.500 + 1.233	56.517 + 3.488	3.578 + 1.376
2000	GS	F	ND	10	12.010 + 1.615	87.800 + 2.586	6.540 + 1.227
1999	GS	F	DS	8	8.838 + 1.884	62.688 + 2.178	3.688 + 0.722
2000	GS	F	DS	10	12.220 + 1.622	80.050 + 3.240	3.390 + 0.654

Port Curtis Mud Crab Shell Disease - nature, distribution and management

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FRDC Project No. 98/210

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Central Queensland University ? Queensland ? Gladstone ? 2001

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Front page muddie courtesy of Pioneer Sea Foods, Gladstone.

PROJECT #98/210
Port Curtis mud crab (*Scylla serrata*) shell disease; nature, distribution and management.

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Objectives:

1. Define the histologic stages of the lesion by developing a sequence of pathological events (includes pathology tests).
2. Define the epidemiology
   a) Define the prevalence and distribution of the disease in each age group in Port Curtis. Determine if the same disease occurs in other areas.
   b) Determine if affected crabs are able to moult and therefore mate successfully by experimentally observing different combinations of diseased male/female crabs. Determine if the crab can shed the ulcerations during a moult. Is there a healing stage?
3. Cross infection: if an infectious agent is isolated/identified, determine its ability to cross infect other species of crustaceans.
4. Depending on the cause/s isolated/identified, work out a management strategy to lessen the effects of rust spot shell disease.
5. Investigate metal burdens of mud crabs from Gladstone compared to other areas.
6. Through the use of copper exposure trials, investigate exposure to metals (in particular copper) as a possible cause of rust spot lesions.

Non Technical Summary:

Outcomes Achieved:
The project outcome that rust spot shell disease in Port Curtis is not infectious has allayed the concerns of commercial and recreational fishing sectors as well as the aquaculture industry, as to the potential impact a contagious disease could have had on these industries. As the project determined that marketability was not affected by gross shell ulcerations, community confidence in the local mud crab fishery has been restored. Although elevated metal concentrations in mud crab tissues are a concern, consumers have been assured that consumption does not pose a potential health risk.

The elevated metal concentrations have, however, raised community awareness of ecosystem health issues, which can occur where there is an interface of urbanisation and fishing habitats. The Queensland Seafood Industry Association has welcomed the release of the results as they highlight the need to maintain a healthy marine environment. The project finding that mud crabs could be “stressed” has alerted managers and relevant agencies to examine current environmental performance indicators. The project has also created extensive knowledge of the epidemiology of shell disease and mud crab biology as a whole.

Commercial fisherman first noticed rust spot shell lesions in the Portunid mud crab (*Scylla serrata* - Forskal) in Gladstone Harbour, Port Curtis Queensland in 1994. The irregular shaped orange coloured lesions commonly called "rust spots" were located on the dorsal shell of the mud crab and had the appearance of cooked crab shell. In advanced cases lesions perforated to form an ulcer, often exposing internal organs. Concerns were raised not only for the potential impact on mud crab marketability, but the possibility that the shell lesions could spread to other crustaceans, in particular the lucrative prawn aquaculture industry. In order to define the disease syndrome, a number of possible causative or contributing factors were examined in the course of the disease investigation. These included infectious/non-infectious causes, environmental factors and contaminant loads.

Over 3000 mud crabs from a number of locations in Queensland (Port Curtis, Ayr, Jacobs Well and Fitzroy River) have been examined for the presence of rust spot shell lesions between 1998 and 2001. The total prevalence of shell lesions in Port Curtis was lower in the 1998/99 sampling (18.3%) compared to that in 2001 (10.2%). Indications from archived crab shells suggest that the prevalence of lesions was much higher when the syndrome was first recorded around 1995. Future samplings should be continued, however, to establish if the decreasing trend continues. There was no significant difference in the prevalence in Port Curtis compared to Fitzroy River, which is possibly due to intermixing of crabs from these two locations. Although lesions occur in mud crabs from Jacobs Well (Moreton Bay) and Ayr, the prevalence is low (from 0-5.6%) compared to Port Curtis. None of the juvenile mud crabs examined from Port Curtis had shell lesions.

A lesion grading system was designed to assist in accurately documenting the area of the shell affected and the severity of lesions. Although females had a higher prevalence of shell lesions than males in Port Curtis in the 1998/99 sampling, lesions occurred in equal frequencies in both sexes in the 1999/00 sampling. Females did, however, have a higher frequency of larger non-perforated lesions than males. There was also a gender difference in the area of the carapace to which lesions were distributed, which may be related to reproduction and spawning in adult females. A majority of affected crabs had two lesions, which were bilaterally symmetrically located on the carapace in over 50% of cases. Less than 10 % of all lesions examined were perforated or ulcerated. As less than 1% of the crabs marketed or consumed from Port Curtis have perforated lesions, it is unlikely that marketability has been affected by the presence of the rust spot syndrome.

Over 60 mud crabs with rust spot lesions were chosen for histopathological examination and the results compared to a reference group of 30 non-diseased mud crabs. There was no evidence of an infectious or parasitic agent being associated with any internal organ or with the carapace lesions. The pathology of the rust spot lesions is restricted to the endocuticle layer (internal shell layer) and adjacent muscle attachment. As this layer is formed after the crab has moulted, it appears that the lesions are caused by a defect in the manufacturing of this layer, while the crab is in the process of calcifying its shell. This pathology contrasts with previously reported pathology of shell diseases in other crustaceans. Here the pathology is an external erosion of the shell, which may be caused by pathogenic organisms, with unsuitable environmental conditions being a contributing factor.

Cross infection trials were conducted in an attempt to transmit rust spot shell disease to other mud crabs, sand crabs and prawns. In water transmission trials, adult and juvenile mud crabs were exposed to Gladstone Harbour water. In inoculation trials, processed tissue from diseased mud crabs was injected into juvenile mud crabs, sand crabs and prawns. None of the treatment groups developed rust spot lesions and after subsequent pathology, no significant lesions were seen in any of the tissues examined. It appears that rust spot lesions do not contain a virus or virus-like organism capable of transmitting the disease to other mud crabs or crustaceans. It is probable that the cause of rust spot shell lesions is non-infectious and possibly environmental.

A number of diseased mud crabs both adult and juvenile was observed through a moulting period in order to determine if lesions could be shed with the old shell when the crab moulted. Old lesions were shed with the old exuviae (shell) and a healed form of the lesion remained in some cases. New lesions were also seen to form in the post moult phase (during production of the new endocuticle) and would most likely remain until the next moult. Although diseased mud crabs even with severe lesions are able to moult successfully and repair shell lesions, in some cases where lesions are extensive, however, they may contribute to the cause of moult death syndrome.

Metal analyses of 220 mud crab tissues were undertaken in 1999 and 2000. Metal concentrations, in particular copper and zinc, were elevated in Gladstone (and Fitzroy River) mud crab hepatopancreas (liver), compared to a reference site (Ayr). There was no significant difference in metal concentrations between the diseased and non-diseased group of mud crabs from Gladstone. The inability to establish differences, however, could be confounded due to the difficulty in assigning a crab to either of these groups being qualitative (the presence of a lesion) rather than quantitative. Concentrations of copper in Gladstone mud crab hepatopancreas were also up to three times higher than concentrations in the hepatopancreas of mud crabs from other locations sampled in Queensland. Metal burdens in Gladstone mud crab hepatopancreas were also elevated compared to Ayr. A high variation in metal concentrations in the Gladstone diseased group of crabs compared to all other groups suggests that these crabs are unable to regulate their metal concentrations and this could indicate some level of stress in this group. Concentrations of all metals in muscle tissue were below those metal concentrations recommended by the Australia New Zealand Food Standards Code (2000) for the consumption of crustacea. This code is continually under review and therefore metal concentrations in mud crab tissues should continue to be monitored. However, in terms of metal concentrations mud crab meat from Gladstone crabs can be considered suitable for consumption.

Through the use of copper exposure trials in which juvenile mud crabs were exposed to sublethal levels of copper, we explored the hypothesis that copper exposure inhibits calcium uptake into the post moult crab shell and could therefore be implicated in the development of rust spot shell lesions. The trial confirmed that calcium uptake into the carapace (shell) of soft-shelled crabs (72 hours post moult) was inhibited by sublethal copper exposure. There was a significant negative relationship between increasing copper concentrations in the hepatopancreas and declining calcium concentrations in the carapace. Several metals including copper have been shown to cause interference with calcium uptake in crustaceans. It is therefore conceivable that exposure to copper, perhaps in combination with other metals/contaminants could be implicated in the cause of rust spot lesions.

Results of crab blood tests in which two immune parameters and one cellular enzyme were measured, suggest that Gladstone crabs have been stimulated to produce higher levels of these immune/cellular factors compared to the Ayr crabs. This could further indicate a higher level of stress in Gladstone crabs compared to those from Ayr. Exposure to pathogens, contaminants and stress are known to effect production of immune factors in aquatic organisms. Although immune factors/cellular enzymes in the female diseased group of crabs from Gladstone were elevated compared to the female crabs from Ayr, they were significantly lower than levels in the non-diseased female crabs from Gladstone. This suggests that production of immune factors/cellular enzymes in this diseased group may have been suppressed or the factors have been destroyed at a faster rate than normal.

Stepwise multiple regressions were used to investigate whether any relationships existed between measures of blood immune/cellular responses and hepatopancreas metal concentrations in Ayr and Gladstone crabs. Results suggest that there is a statistical relationship between the metal concentrations and immune/cellular responses. There were fewer, weaker relationships identified in the Ayr crabs in comparison to the Gladstone groups, although at times relationships in the Gladstone crabs were inhibitory rather than stimulatory (i.e. indicating exposure to a metal causing inhibition rather than stimulation of a blood parameter). Although the regressions do not prove cause and effect they do, however, provide proof of a relationship, whereby blood parameters can change as a function of a change in metal accumulations in Gladstone mud crab tissues.

The results of metal analyses of water and sediments from the permanent burrows of Gladstone mud crabs, indicate that fairly low concentrations of metals exist in the burrows and therefore these sites are unlikely to be a source of elevated metals in Gladstone mud crabs. A pilot study using stable isotopes of carbon and nitrogen as an alternative to gut content analyses was used to determine if differences existed in the diets of Gladstone crabs compared to Ayr crabs which, might explain the contrasting tissue metal results between the two sites. The results although preliminary, suggest that the Gladstone crabs may be consuming something in their diet, which is enriched in copper but is not available in the Ayr mud crab diet.

Our findings have raised questions as to the comparative "health" of Gladstone crabs compared to Ayr and hence the quality of the ecological environment of Port Curtis. As the cause of mud crab rust spot shell lesions appears to be a local environmental one, the source of elevated metal concentrations especially copper and zinc, needs to be investigated further. The impact of elevated metal burdens and stimulated immune responses and cellular enzymes on individual mud crabs is not known. The Centre for Environmental Management (CQU) is conducting two small research projects to determine metal concentrations in other biota in Port Curtis which may be part of the mud crab diet. There is, however, an urgent need for research to go beyond the food sources of the mud crab, to the origin of the contamination of those organisms eg. urban, industrial or agricultural.

Our recommendations are:

1. To establish if elevated metal concentrations exist in other biota in Port Curtis.
2. Determine the source of elevated metal concentrations.
3. Reduce the flow of metals into Port Curtis from all sources by reviewing the current point and area sources of discharge.

4. Continue to monitor crab health in terms of the prevalence of rust spot shell lesions and metal burdens, ensuring metal levels remain within recommended levels.

Further research into the ecosystem health of Port Curtis will determine what management strategies are required to address this issue.